The Endothelial Cell Protein C Receptor

INHIBITION OF ACTIVATED PROTEIN C ANTICOAGULANT FUNCTION WITHOUT MODULATION OF REACTION WITH PROTEINASE INHIBITORS*

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The Influence of EPCR on Plasma Clotting—The anticoagulant activity of APC in plasma was determined in a one-stage clotting assay using the ST4 coagulation instrument (Diagnostica Stago, Parsippany, NJ). The factor Xa concentration was adjusted to give a 30-s clotting time in a mixture containing 20 μl of factor Xa, 20 μl of CaCl₂ (6.25 mM) and MgCl₂ (0.6 mM), and 10 μl of phospholipid vesicles (final 0.1 mg/ml of 80% PC and 20% PS, w/v). APC (20 μl to give a final concentration of 57 μl in the assay) and s-EPCR-HPC4 (80 μl) were added prior to the addition of plasma. Clotting was initiated with 50 μl of pooled human plasma.

EXPERIMENTAL PROCEDURES

Materials—Phospholipid vesicles (14), bovine prothrombin and thrombin (15), bovine factor V (12), human protein C (16), human and bovine APC (16), bovine protein S (17), bovine factor Xa (18), rabbit thrombomodulin (19), and soluble recombinant EPCR truncated at residue 210 to remove the transmembrane domain and the cytoplolic tail with the HPC4 epitope on the carboxyl terminus (s-EPCR-HPC4) (35) were isolated as described.

The protein C anticoagulant pathway functions in large part on the endothelial cell surface (reviewed in Refs. 1, 2). The pathway is initiated when thrombin binds to thrombomodulin on the endothelium, and this complex catalyzes protein C activation. Activated protein C (APC) can interact with protein S on the surface of endothelium or other cells to inactive factors Va and Vithe (3–6). Deficiencies in components of this system lead to thrombophilia (reviewed in Refs. 7, 8). APC is slowly inactivated in plasma and in vivo primarily by the plasma proteinase inhibitors, α₁-antitrypsin and protein C inhibitor (9). Recently we identified an endothelial cell surface glycoprotein, EPCR (10), that binds directly to protein C and APC with high affinity even when expressed as a soluble form (35). The physiological function of EPCR remains unknown.

A soluble form of the endothelial cell protein C receptor (EPCR) was analyzed for the ability to modulate the functional properties of protein C and activated protein C (APC). In a plasma clotting system initiated with factor Xa, EPCR blocked the anticoagulant activity of APC in a dose-dependent fashion. EPCR had no influence on clotting in the absence of APC. Consistent with the plasma results, EPCR slowed the proteolytic inactivation of factor Va by slowing both of the key proteolytic cleavages in the heavy chain of factor Va. EPCR did not prevent protein C activation by the soluble thrombin-thrombomodulin complex, did not alter the inactivation of APC by α₁-antitrypsin or protein C inhibitor, and did not influence the kinetics of peptide paranitroanilide substrate cleavage significantly. We conclude that EPCR binds to an exosite on APC that selectively modulates the enzyme specificity in a manner reminiscent of the influence of thrombomodulin on thrombin.

The goat polyclonal antibody to the factor Va heavy chain was affinity purified over a 2-ml Affi-Gel 15 factor Va heavy chain column (3–mg of heavy chain/ml of resin) prior to use in the Western blot analysis. Antibody was eluted from the column with 0.1% glycine, pH 2.1, and fractions were immediately neutralized with 1 volume of Tris-HCl, pH 8.0.

The influence of EPCR on plasma clotting—The anticoagulant activity of APC in plasma was determined in a one-stage clotting assay using the ST4 coagulation instrument (Diagnostica Stago, Parsippany, NJ). The factor Xa concentration was adjusted to give a 30-s clotting time in a mixture containing 20 μl of factor Xa, 20 μl of CaCl₂ (6.25 mM) and MgCl₂ (0.6 mM), and 10 μl of phospholipid vesicles (final 0.1 mg/ml of 80% PC and 20% PS, w/v). APC (20 μl to give a final concentration of 57 μl in the assay) and s-EPCR-HPC4 (80 μl) were added prior to the addition of plasma. Clotting was initiated with 50 μl of pooled human plasma.
Influence of EPCR on Factor Va Inactivation—APC cleavage and inactivation of factor Va was assayed as follows. Bovine factor Va (200 nM) was incubated with APC (2 nM) in the presence or absence of s-EPCR-HPC4 (1 nM) in 0.1 M NaCl, 0.02 M Tris- HCl, pH 7.5, 3 mM CaCl₂, and 0.6 mM MgCl₂ containing PS/PC/PE vesicles (20:40:40, 0.1 mg/ml) at 37 °C. At the indicated time points, samples were removed, diluted 1:10 with 20 mM Tris- HCl, pH 7.5, 0.1 M NaCl containing 0.1% gelatin. Factor Va activity was immediately assessed with a one-stage clotting assay in factor V-deficient plasma as described previously (12). Clot formation was detected using a Diagnostica Stago ST4. Assays were performed at 37 °C. Factor Va activity was determined based on reference standard curve of normal bovine plasma. A separate sample was also subjected to SDS-PAGE.

Protein Sequencing—Amino-terminal sequence analysis of factor Va proteolytic fragments electrophoresed onto a polyvinylidene difluoride membrane was performed in Dr. Kenneth Jackson's laboratory (Molecular Biology Research Facility, St. Francis of Tulsa Medical Research Institute, Oklahoma City).

The Influence of EPCR on APC Amidolytic Activity—The effect of s-EPCR-HPC4 on the amidolytic activity of APC was performed by preincubating 10 nM APC and 200 nM s-EPCR-HPC4 in 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl₂, 0.6 mM MgCl₂, 0.1% (w/v) gelatin, pH 7.5, for 15 min at room temperature. A 50-μl aliquot of reaction solution was mixed with 500 μl of substrate (0–1 mM final) on a microtiter plate, and the change in absorbance with time at 405 nm was determined on a Vmax microplate reader (Molecular Devices). All samples were assayed in duplicate. The concentration of APC was determined by reference to a standard curve prepared with known concentrations of freshly prepared APC.

The Influence of EPCR on Protein C Activation—Human protein C (0.2 μM) was incubated with rabbit thrombomodulin (10 nM) and bovine thrombin (0.2 nM) in the presence and absence of 500 or 2000 nM s-EPCR-HPC4. The buffer contained 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/ml gelatin, with either 3 mM CaCl₂ or 3 mM CaCl₂ and 0.6 mM MgCl₂. The protein C and s-EPCR-HPC4 were preincubated for 15 min at room temperature, thrombomodulin was added, and activation was initiated by the addition of thrombin. After 15 min at 37 °C, the reaction was stopped by addition of 100 μg/ml antithrombin III. APC formed during the activation was determined by amidolytic activity with Spectrozyme PCAs as the substrate (0.4 mM) in the above buffer without CaCl₂ or MgCl₂. The concentration of APC was determined by reference to a standard curve prepared with known concentrations of freshly prepared APC.

The Effect of s-EPCR-HPC4 on the Inhibition of APC by Serpins—The inhibition of activated protein C by the protein C inhibitor was performed by preincubating activated protein C (10 nM) with or without 1 μM s-EPCR-HPC4 in 20 mM Tris-HCl, 0.1 M NaCl, 0.1% (w/v) gelatin, 10 μg/ml heparin, 3 mM CaCl₂, 0.6 mM MgCl₂, pH 7.5, for 10 min at room temperature. Inhibition was begun by the addition of recombinant protein C inhibitor to a final concentration of 60 nM in 50-μl final volume. At various times, 50 μl of 0.8 mM Spectrozyme PCa was added, and the absorbance increase in absorbance with time at 405 nm was read on a Vmax microplate reader. All samples were done in duplicate, and at least 50% inhibition was achieved for calculation of inhibition rates. The second order rate constant (kₐ, m⁻¹ min⁻¹) was calculated at each time point, and the data are presented as the average ± standard deviation.

The Effect of s-EPCR-HPC4 on the Inhibition of APC by α₁-antitrypsin was determined at 37 °C with 340 nM APC and 6.2 μM inhibitor in the presence or absence of 380 nM s-EPCR-HPC4 in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM CaCl₂, 0.6 mM MgCl₂, 1 mg/ml bovine serum albumin, 0.02% NaN₃. At time intervals from 70 to 260 min, samples were removed and assayed for residual APC amidolytic activity using Spectrozyme PCa as the substrate (0.4 mM). Greater than 50% inhibition was achieved for all samples containing α₁-antitrypsin.

Electrophoresis—Electrophoresis was performed according to the method of Laemmli (22) using 10% polyacrylamide gels. Samples were incubated in 2% SDS buffer for 3 min at 100 °C prior to loading. Gels were electrophoresed at 190 V for 45 min. Protein was transferred to polyvinylidene difluoride membranes using a Bio-Rad semi-dry transfer apparatus at 6 V for 10 min. After blocking the membranes with non-fat dry milk, the membranes were incubated with goat polyclonal antibody to the factor Va heavy chain for 30 min at room temperature with gentle agitation. The blot was washed three times (5 min each) with 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, containing 0.05% Tween 20. The secondary antibody used was a biotin-conjugated donkey anti-goat polyclonal (0.1 μg/ml) for 30 min. The blot was washed as above. After incubation with alkaline phosphatase-conjugated streptavidin (0.1 μg/ml) for 30 min, the bands were detected with the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazoli-

**Fig. 1.** s-EPCR-HPC4 inhibits the anticoagulant activity of APC. A, the anticoagulant activity of APC was determined in a modified factor Xa assay as described under "Experimental Procedures." B, s-EPCR-HPC4 was added at the indicated final concentrations in the presence of 57 nM APC (closed circles) or no added APC (open circles). Densitometric scans were performed using a Bio-Rad Imaging Densitometer (GS-670) and Molecular Analyst software.

**RESULTS**

The observation that s-EPCR-HPC4 binds APC in solution with relatively high affinity (Kd(app) = 29 nM) allowed us to examine the influence of complex formation on APC anticoagulant activity. As anticipated, as the APC concentration was increased, the clotting time in a factor Xa one-stage assay increased linearly (Fig. 1A). Holding the APC concentration constant (57 nM), the clotting time decreased progressively as a function of increasing s-EPCR-HPC4 concentration until nearly all of the APC anticoagulant activity was eliminated (Fig. 1B). The presence of s-EPCR-HPC4 had no effect on the clotting times in the absence of APC. Half of the APC anticoagulant activity was blocked with s-EPCR-HPC4 concentrations between 100 and 200 nM. Assuming that the s-EPCR-HPC4 formed an inactive complex with APC, the free APC concentration was calculated from the standard curve in Fig. 1A. A KD(app) of 168.4 ± 85.1 nM for s-EPCR-HPC4 was obtained from these data. This value was calculated from s-EPCR-HPC4 concentrations ranging from 50 to 1000 nM. Thus, the concentration required to inhibit the APC anticoagulant effect in plasma is much higher than the Kd(app) observed for the binding interaction with APC. This would be anticipated if s-EPCR-HPC4 competed with a plasma protein for binding to APC. The ~20 nM protein C present in the plasma could not account for these differences even if the plasma protein C could
partially displace the APC during the clotting assay.

Based on current concepts of blood coagulation, of the known substrates for APC, only factor Va is involved in controlling the factor Xa one-stage clotting time. Since s-EPCR-HPC4 blocked APC anticoagulant activity in plasma, we assumed that the effect was due to inhibition of APC-mediated factor Va inactivation. As expected, s-EPCR-HPC4 slowed APC inactivation of factor Va at least 10-fold (Fig. 2). At equivalent concentrations of APC and factor Va, it required 4 min to inactivate approximately 60% of the factor Va activity in the absence of s-EPCR-HPC4 and approximately 60 min to inactivate comparable amounts of factor Va in the presence of s-EPCR-HPC4. To determine which steps in factor Va inactivation were influenced by s-EPCR-HPC4, we analyzed the time course of the factor Va inactivation by SDS-PAGE. Since the cleavage sites responsible for factor Va inactivation are in the heavy chain, and cleavage of the heavy chain by APC generates bands that migrate with the light chain, we employed Western blot analysis with polyclonal anti-heavy chain antibody to simplify the interpretation. s-EPCR-HPC4 slowed the formation of all of the proteolytic inactivation products with no obvious change in the relative distribution of the products (Fig. 3A). The inhibition of APC activity correlated with the inhibition of cleavage of factor Va heavy chain. APC cleaved factor Va at Arg-505 to give the fragments labeled a and b and at Arg-306 to give the fragment c or at both sites to give the fragments c and e. Since the protein bands tended to run with higher than anticipated apparent molecular mass in this gel system, the cleavage products were confirmed by amino-terminal sequencing. The a and c fragments both had the amino-terminal sequence of bovine factor Va. The sequence of fragment b began at amino acid 506. The sequence of fragment e began at residue 307. Fragment d could not be identified unambiguously since it failed to give amino-terminal sequence on two occasions. Densitometric scanning of the Western blots (Fig. 3B) indicated that in the reaction containing s-EPCR-HPC4, the rate of cleavage of the intact factor Va heavy chain was reduced by 10-fold. To standardize each lane for variations in loading, the ratio of intact factor Va heavy chain to intact heavy chain plus fragment b was plotted for each point.

When these reactions were performed as above in the presence of 10 nM protein S, factor Va was inactivated slightly faster in both cases, with 50% activity remaining at 3 min in the absence and 30 min in the presence of s-EPCR-HPC4 (data not shown). Therefore, protein S does not appear to interfere with the interaction between s-EPCR-HPC4 and APC.

In the presence of the membrane surface, the local factor Va and APC concentrations are high. Therefore, if the factor Va and s-EPCR-HPC4 had overlapping binding sites, the IC50 for s-EPCR-HPC4 required for inhibition would be higher than the Kd(app) obtained from competition studies in the absence of factor Va. We examined the APC concentration dependence of factor Va inactivation in the absence of s-EPCR-HPC4 (Fig. 4A) and then titrated increasing concentrations of s-EPCR-HPC4 into the reaction (Fig. 4B). Assuming that the inhibition of APC activity was due to complex formation with s-EPCR-HPC4, we calculated an IC50 = 814 ± 79 nM. The data in Fig. 4B are fitted assuming that the complex has no anticoagulant activity. The fit appears reasonable, so within the limits of our current capacity it appears that the complex has little or no activity. The complexity of the system, including the fact that the inactivation complex is membrane bound and that inactivation involves multiple steps, each of which contributes to loss of activity, makes a detailed kinetic analysis of the inhibition
These studies are consistent with the active site being accessible to very small substrates but do not exclude the possibility that s-EPCR-HPC4 inhibits factor Va inactivation by blocking the extended substrate binding pocket. The chromogenic substrates do not interact on the prime side of the primary specificity pocket. Therefore, we investigated the influence of s-EPCR-HPC4 on the inhibition rate of APC by two physiologically relevant serpin-type proteinase inhibitors, protein C inhibitor and α1-antitrypsin. Serpins interact on both the P and P′ side with contacts extending to at least 4 residues on the prime side in the cases that have been examined (23). The protein C inhibitor activity was essentially independent of the presence of s-EPCR-HPC4 under conditions known to support the enzyme-receptor binding. The second order rate constant (k2, M−1min−1) in buffer containing 10 μg/ml heparin was 1.24 ± 0.53 × 108 for APC alone and 1.08 ± 0.27 × 108 for APC in the presence of a 100-fold molar excess of s-EPCR-HPC4. These constants are similar to published values using the recombinant inhibitor (24), and 10 μg/ml heparin did not inhibit the binding of fluorescently labeled APC to 293T cells transfected with full-length human EPCR as determined by fluorescence-activated cell sorter analysis (data not shown). In a similar fashion, s-EPCR-HPC4 had little effect on the ability of α1-antitrypsin to inhibit APC. After 140 min incubation at 37 °C, 48.1 and 56.4% of the APC was inhibited by α1-antitrypsin in the presence or absence of s-EPCR-HPC4, respectively.

Since s-EPCR-HPC4 inhibited factor Va inactivation, we examined the possibility that it might also modulate protein C activation. Activation of protein C with the soluble thrombin-thrombomodulin complex was not altered by the presence of s-EPCR-HPC4. In these experiments, the thrombin-thrombomodulin complex generated 2.13 nM APC in the absence, 2.45 nM APC in the presence of 500 nM s-EPCR-HPC4, and 2.69 nM APC in the presence of 2 μM s-EPCR-HPC4 when the reaction was performed as described under “Experimental Procedures” in the presence of Ca2+-containing buffers. With Ca2+ and Mg2+ present, the complex generated 2.46 nM APC in the absence of s-EPCR-HPC4, 2.29 nM APC in the presence of 500 nM s-EPCR-HPC4, and 2.44 nM APC with 2 μM s-EPCR-HPC4. The protein C concentration employed is below K0.2 for the reaction (25), and thus it seems unlikely that the soluble complex can influence any of the kinetic parameters of protein C activation.

**DISCUSSION**

The central goal of this study was to examine the influence of EPCR on APC specificity. Our working hypothesis is that EPCR might modulate APC specificity in a manner analogous to the specificity changes observed when thrombin complexes with thrombomodulin. In this regard, the present study reflects many similarities in the two systems. Thrombomodulin blocks the ability of thrombin to cleave its natural substrates, factor V (26), factor XIII (27), fibrinogen (26), and platelets (28). EPCR prevents inactivation of factor Va by APC. Neither receptor blocks chromogenic activity of their respective enzymes. Both enzyme-receptor complexes react with proteinase inhibitors essentially equivalently, or in some cases better, than the free enzyme (29). Both receptors bind their respective enzymes after the modification with peptide chloromethyl ketones that extend into the S4 pocket. A major difference is that thrombomodulin accelerates protein C activation by thrombin, but as yet we have not identified a substrate that is positively influenced by s-EPCR-HPC4 complex formation with APC. Thus, these two systems share several common features and appear to have the active site open from the S4 to the S4′ substrate binding pocket. Thus, s-EPCR-HPC4 must be inhibiting APC-dependent factor Va inactivation by binding to an exosite.

**FIG. 4.** Concentration dependence of s-EPCR-HPC4 inhibition of factor Va inactivation. A, Factor Va (200 nM) was incubated with the indicated concentrations of APC in 20 mM Tris, pH 7.5, 0.1 mM NaCl, 3 mM CaCl2, 0.6 mM MgCl2, and PS/PC/PE vesicles (0.1 mg/ml) for 5 min at room temperature. Samples were diluted 200-fold and incubated with prothrombin (200 nM) and factor Xa (1 mM) in the above buffer containing PS/PC/PE vesicles (0.1 mg/ml). Reaction mixtures were incubated at room temperature for 2 min. EDTA (50 mM final concentration) was added to stop the reaction. Substrate S-2238 was added to 1 mM, and the samples were read for 2 min at 405 nm using a Vmax reader to measure thrombin formation. B, Factor Va (200 nM) and APC (10 nM) were incubated with the indicated concentrations of s-EPCR-HPC4 and assayed as above. Residual APC activity was determined using the linear portion of the standard curve (0–10 nM) constructed in A. Percent residual activity was plotted against s-EPCR-HPC4 concentration, and data were fitted using the equation Y = A[B/(B + X)], where A is 100% APC activity (i.e. no EPCR), B is the IC50, and X is the EPCR concentration. The data were fit to this equation with the Enzfitter program (R.J. Leatherbarrow, Elsevier Biosoft).
Thrombomodulin inhibition of fibrinogen clotting appears to be due primarily to the fact that fibrinogen and thrombomodulin both interact with anion binding exosite 1 and hence compete for binding to thrombin (30–33). As a working model, one might question whether EPCR might interact with an anion exosite on APC similar to that on thrombin. This possibility is supported by the structure of APC. Recently, we solved the structure of a proteolytic fragment of APC lacking the Gla domain to 2.8 Å (34). APC has a deep groove on the prime side of the active site that is reminiscent of anion binding exosite 1 of thrombin. The groove of APC has basic residues lining the rim and hydrophobic residues at the base. Thus, thrombin and APC share structural features in common that might contribute to the specificity changes observed with EPCR. Current studies are directed toward the potential involvement of this putative exosite in EPCR binding.

Physiologically, it is unlikely that EPCR functions by inhibiting factor Va inactivation. The receptor has a low copy number, and preliminary studies indicate that it is not present on many vessels. Thus, only a small percentage of APC or protein C would be anticipated to be bound to this endothelial cell receptor. It remains possible that EPCR inhibition of APC-mediated factor Va inactivation is due to the use of a soluble form of EPCR. This possibility will have to be excluded by alternative strategies. The fact that both EPCR and thrombomodulin are expressed on the same membrane surface raises the possibility that EPCR may present protein C to the activation complex on the cell surface and then hold the product to perform as yet unidentified functions.

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