Reconstitution of the Human Endothelial Cell Protein C Receptor with Thrombomodulin in Phosphatidylcholine Vesicles Enhances Protein C Activation*

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Blocking protein C binding to the endothelial cell protein C receptor (EPCR) on the endothelium is known to reduce protein C activation rates. Now we isolate human EPCR and thrombomodulin (TM) and reconstitute them into phosphatidylcholine vesicles. The EPCR increases protein C activation rates in a concentration-dependent fashion that does not saturate at 14 EPCR molecules/TM. Without EPCR, the protein C concentration dependence fits a single class of sites ($K_m = 2.17 \pm 0.13 \mu M$). With EPCR, two classes of sites are apparent ($K_m = 20 \pm 15 \mu M$ and $K_m = 3.2 \pm 1.7 \mu M$). Increasing the EPCR concentration at a constant TM concentration increases the percentage of high affinity sites. Holding the TM: EPCR ratio constant while decreasing the density of these proteins results in a decrease in the EPCR enhancement of protein C activation, suggesting that there is little affinity of the EPCR for TM. Negatively charged phospholipids also enhance protein C activation. EPCR acceleration of protein C activation is blocked by anti-EPCR antibodies, but not by annexin V, whereas the reverse is true with negatively charged phospholipids. Human umbilical cord endothelium expresses approximately 7 times more EPCR than TM. Anti-EPCR antibody reduces protein C activation rates 7-fold over these cells, whereas annexin V is ineffective, indicating that EPCR rather than negatively charged phospholipid provide the surface for protein C activation. EPCR expression varies dramatically among vascular beds. The present results indicate that the EPCR concentration will determine the effectiveness of the protein C activation complex.

The protein C anticoagulant pathway is critical to the negative regulation of the blood clotting cascade (for review, see Refs. 1 and 2). The pathway is initiated when thrombin binds to thrombomodulin (TM), an integral membrane protein on endothelium and some other cell types. This complex then converts protein C to activated protein C (APC), which, in complex with protein S, inactivates factors Va and VIIIa resulting in inhibition of thrombin generation.

Protein C is a vitamin K-dependent zymogen that, like other vitamin K-dependent proteins, is capable of binding to negatively charged phospholipid surfaces (3, 4). Unlike the other vitamin K-dependent proteins, activation occurs on the surface of endothelial cells. In vivo, endothelial cells serve primarily anticoagulant functions. Because negatively charged phospholipid would serve to augment the clotting cascade as well as the anticoagulant pathway, for the endothelium to serve selective anticoagulant functions alternative mechanisms are required for assembling anticoagulant complexes on the endothelial cell surface. Endothelial cells constitutively express a protein C/APC-binding protein, designated the endothelial cell protein C receptor (EPCR) (5), which appears to serve this function. Blocking protein C binding to EPCR decreases protein C activation by the thrombin-TM complex on cultured endothelium, primarily because of an increase in the $K_m$ for protein C (6). In humans, the plasma concentration of protein C, ~65 nM, is considerably below the $K_m$ for the thrombin-TM activation complex incorporated into phosphatidylcholine vesicles (2 $\mu M$ (7)).

Because the vasculature varies dramatically in diameter between the large vessels and the capillaries, assuming a constant TM density per endothelial cell, the effective TM concentration rises from less than 5 $\mu M$ to more than 100 $\mu M$ as the blood moves from the aorta to the capillaries (1, 8). This has led to the concept that most protein C activation occurs in the microcirculation (1). These considerations raise the question of whether there might be a compensatory mechanism to augment protein C activation selectively in the large vessels. EPCR expression varies throughout the vasculature (9) and is sensitive to a variety of effector systems (5). In vivo, EPCR expression levels are highest on the endothelium of large vessels, decreasing progressively with decreasing vessel size until the EPCR becomes undetectable by immunohistochemical approaches in most capillary beds (9, 10). With cultured endothelium, EPCR expression is down-regulated by cytokines like tumor necrosis factor $\alpha$ (5, 11). In contrast, EPCR mRNA levels are up-regulated by thrombin in cell culture and by endotoxin and thrombin in vivo (12). Because of the variable EPCR expression on endothelium, the relationship between EPCR expression levels and its cofactor function in protein C activation gains biological importance for understanding the anticoagulant properties of different vascular beds.

To address the relationship between the EPCR concentration...
and protein C activation, we have isolated EPCR from placenta and recombinant EPCR from cultured cells and reconstituted the protein into phospholipid vesicles. The results indicate that an excess of EPCR to TM is required for optimal EPCR enhancement of protein C activation and that the EPCR enhances activation effectively when reconstituted into phosphatidylcholine vesicles that cannot effectively support the coagulation cascade.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human protein C and APC (13), recombinant Gla-domainless protein C (rGDPC) (14), bovine (15) and human (16) thrombin, monoclonal antibodies (mAbs) against human EPCR (1494, 1495, 1496, 1500) (6), mAbs against human TM (CTM109, CTM1029) (13), and phospholipid vesicles (17) were prepared as described. Recombinant annexin V was a generous gift from Dr. Kazuo Fujikawa. The following reagents were purchased from the indicated vendors: Spectrozyme PCa, American Diagnostic Inc.; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine, and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine, Avanti Polar Lipids, Inc.; [3H]phosphatidylcholine, NEN Life Science Products; iodine-125, Amersham Pharmacia Biotech; octyl-O-glucopyranoside (OG), Calbiochem; Lubrol PX, Triton X-100, chondroitin ABC, Sigma; Affi-Gel 10, Bio-Rad.

**Constants Employed for Determination of Protein Concentrations**—The extinction coefficients, ε 1 cm at 280 nm, and molecular weights used for this study were: human protein C (14, 62,000) and APC (14.5, 61,000) (18), rGDPC (13.7, 58,000) (14), human TM (10, 75,000), EPCR (10, 35,000) (19), bovine (21, 57,000) (15) and human (17.5, 36, 400) (20) thrombin.

**Cell Culture**—Human kidney 293 cells were stably transfected with human EPCR (referred to as E7 cells) and cultured as described previously (19). Human umbilical vein endothelial cell(s) (HUVEC) were cultured with endothelial cell growth factor, heparin, and heat-inactivated bovine serum as described (21).

**Purification of EPCR and TM from Placenta**—All purification procedures up to and including immunoaffinity purification were performed at 4 °C. Two frozen human placentas were thawed, diced, and passed through a commercial, motor-driven meat grinder (Leeson Electric Co., Grafton, WI) five times. The ground placenta was suspended in 1,600 ml of 0.25 m sucrose, 5 mM benzamidine-HCl, 0.02% NaN3, 5 mM M NaCl, 20 mM HEPES-HCl, pH 7.5. Larger particles were partially disrupted by repeatedly drawing the sample into a 60-ml catheter syringe followed by rapid sample expulsion. The tissue was collected by centrifugation at 4,800 × g for 30 min. The pellets were resuspended and collected by centrifugation as before, except that the chloromethyl ketone was omitted in the wash buffer and thereafter. This process was repeated two times. EPCR and TM were extracted from the washed pellet with 2 volumes and thereafter. This process was repeated two times. EPCR and TM were extracted from the washed pellet with 2 volumes of wash buffer made 1% in Triton X-100 using a Tissumizer Mark II (Tekmar Company, Cincinnati, OH) homogenizer. Insoluble material was removed by centrifugation at 40,000 × g for 30 min, and particulates were removed with a 0.45 μm filter. The filtrate was applied to the anti-EPCR mAb 1496 Affi-Gel 10 column (8 ml) at 0.67 ml/min. The column was washed and eluted essentially as described above for the placental EPCR, except that the volumes were reduced in proportion to the resin volume. The protein-containing fractions were pooled, concentrated, and buffer exchanged into TBS containing 15 mM OG using the Centricon-30.

**Reconstitution of EPCR and TM into Phospholipid Vesicles**—Purified EPCR and TM were incorporated into phospholipid vesicles by minor modifications of the method described by Galvin et al. (22). Briefly, 1 mg of phospholipid was dissolved with 40 μl of 14% OG in TBS. TM (5 μg) and EPCR ranging from 0.15 to 2.0 μg/ml were added to each volume, which was adjusted to 200 μl with TBS. [3H]Phosphatidylcholine was included as a tracer. The samples were dialyzed against three changes, 1 liter each, of TBS, 0.02% NaN3, for 45 h. The dialyzed sample was mixed with 0.2 ml of 50% sucrose, overlaid with 0.8 ml of 20% sucrose, and finally overlaid with 0.2 ml of TBS before centrifugation at 20 °C in a TLA-100.2 rotor (Beckman) for 16 h at 100,000 × g. 0.2-ml fractions were collected from the sucrose density gradients. The phospholipid concentration was determined based on [3H]phosphatidylcholine content (22). The surface-expressed TM concentration was determined based on the rate of rGDPC activation and compared with a standard curve with purified TM as described (22) (also see below). Previous studies have shown that the activation of GDPC is not influenced by incorporation of TM into liposomes (22) or by the presence of EPCR (6).

**ELISA for EPCR**—Protein C or GDPC Activation Assay—Protein C and GDPC activation rates by liposome-incorporated TM were measured by a modification of the method of Galvin et al. (22). Briefly, vesicles containing 20% phosphatidylcholine and TM and EPCR were mixed at 1:10 in a 96-well microtiter plate with 0.1 μg protein C or rGDPC in Hanks’ balanced salt solution containing 0.1% bovine serum albumin, 3 mM Ca2+, and 0.6 mM Mg2+. 5 mM human or bovine thrombin was added to initiate activation. In some cases, 0.1 μg anti-EPCR mAb 1494 or 1 μg annexin V was added before protein C and preincubated with the vesicles for 5 min at room temperature to block the EPCR or to block the negatively charged phospholipid head groups. The samples were incubated at defined time intervals by the addition of 0.20 volume of antithrombin (1.5 mg/ml) containing 20 μl TBS, 20 mM HEPES-HCl, pH 7.5. APC and activated rGDPC concentrations were determined based on their amidolytic activities toward 0.2 mM Spectrozyme PCa substrate in 0.15 M NaCl, 20 mM HEPES-HCl, pH 7.5. The rates of substrate cleavage were measured with a T m-modified plate reader (Molecular Devices). The concentration of enzyme in these reaction mixtures was determined by comparison with a standard curve of amidolytic activity versus enzyme concentration constructed with freshly prepared, fully activated protein C or rGDPC (22). Under the conditions employed in this study, less than 10% of the Protein C was activated during the assay, and all assays were linear as a function of time between the initiation and termination of the assay.

**ELISA for EPCR**—The assay was modified from Kurosawa et al. (23). Microtiter plates were coated overnight at 4 °C with 50 μl of 5 μg/ml anti-EPCR mAb 1949 in 15 mM NaCO3, 35 mM NaHCO3, pH 9.6. The remaining steps were performed at room temperature. The wells were washed three times containing 0.05% Tween 20 and were coated with 0.025 μg/ml of a 1 μg/ml standard, 50-μl samples in TBS containing 0.05% Tween 20 were added in duplicate wells, and the plates were incubated for 1 h. The wells were washed and 50 μl of biotin-conjugated anti-EPCR mAb 1949 (2 μg/ml) or biotin-conjugated anti-EPCR mAb 1,500 (1 μg/ml) was added. The plates were incubated for 1 h, washed, and 50 μl of streptavidin–alkaline phosphatase conjugate (1 μg/ml) was added and incubated for 1 additional h. The wells were washed five times, and 50 μl of 1 mg/ml p-nitrophenyl
phosphate substrate was added. The end point absorbance at 405 nm was read on a Vmax microplate reader. The standard curve was linear (r = 0.999) from 0 to 100 ng/ml.

Chondroitinase Treatment of TM and Western Blotting—TM (0.5 μg/ml) in TBS containing 0.1% Labrol PX was incubated for 1 h with 0.5 unit/ml chondroitinase ABC at 37 °C before analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (24). For Western blot analysis, gels were transferred to polyvinylidene difluoride membranes (Millipore) in a semi-dry apparatus (Bio-Rad). Membranes were blocked with non-fat milk, incubated with anti-TM mAb CTM1009, washed, and incubated with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The membranes were washed and developed with the ECL substrate (Amersham Pharmacia Biotech).

Preparation and Iodination of mAb Fab Fragments—Anti-EPCR mAb 1500 (1.2 mg/ml) and anti-TM mAb CTM1029 (3.3 mg/ml) in phosphate-buffered saline were added to 7.5 and 30 μl cysteine, respectively. The mAbs were then incubated at 37 °C for 90 min with a 1:50 ratio (w/w) of ficin (Sigma). Iodoacetamide (11.25 mM for mAb 1500) was added to inhibit further proteolysis. The digests were buffer exchanged into TBS on a PD-10 column (Amersham Pharmacia Biotech). The supernatants were transferred to the 96-well microplate, and the labeled Fab fragments were separated from free iodine on a PD-10 column according to the manufacturer’s protocol. 125I-Labeled Fab fragments were separated from free iodine on a PD-10 column.

Estimation of EPCR and TM Expression on HUVEC—Confluent HUVEC in 24-well plates were washed two times with wash buffer (Hanks’ buffered salt solution containing 0.1% bovine serum albumin, 3 mM CaCl2, 0.6 mM MgCl2). Binding studies were carried out in reaction mixtures containing medium 199 supplemented with 0.5% bovine serum albumin and 0.1% normal human IgG in a total volume of 0.2 ml. After incubation of cells with 125I-labeled anti-EPCR (1500) or anti-TM mAb CTM1029 Fab fragments (30 ng/ml) at 37 °C for 30 min, the cells were washed rapidly four times with cold wash buffer. The Fab fragments were then eluted from the cells three times with cold acid buffer (0.15 M NaCl, 20 mM glycine-HCl, pH 2.5). 125I-L in the supernatant was measured in an Iso-Data 500 γ-counter. The number of Fab binding sites was calculated based on the radioactivity in the supernatant and the specific activity of the 125I-labeled mAb Fab fragments. Nonspecific binding was determined with a 100-fold excess of the corresponding unlabeled mAb and was less than 5% of the total counts eluted.

Protein C or GDPC Activation Assay on HUVEC—HUVEC in 24-well plates were washed two times with wash buffer and preincubated for 5 min with wash buffer, 0.1 μM 1494, 0.1 μM CTM1009, or 1 μM recombinant annexin V before the addition of 0.1 μM protein C or rGDPC. The activation reactions were initiated by addition of 5 nM bovine thrombin in a total volume of 0.2 ml. After 30 min at 37 °C, the reactions were stopped by adding 40 μl of antithrombin (1.66 mg/ml) to the reactions. The supernatants were transferred to the 96-well microplate, and the amidolytic activities of activated protein C or rGDPC were determined toward 0.2 mM Spectrozyme PCa substrate in 0.15 M NaCl, 20 mM HEPES-HCl, pH 7.5. APC or activated rGDPC concentrations were determined by reference to a standard curve as described above under “Protein C or GDPC Activation Assay.” All determinations were performed in duplicate.

RESULTS

Human TM and EPCR were isolated from placenta as described under “Experimental Procedures.” The SDS-PAGE of freshly prepared EPCR is shown in Fig. 1A and exhibits one major band with or without disulfide bond reduction. EPCR stored at 4 °C formed some aggregates that were partially eliminated by disulfide bond reduction (Fig. 1B), consistent with the aggregation being mediated by the free Cys residues in the extracellular domain, the transmembrane domain, and the cytosolic tail of the EPCR (5, 19). EPCR from placenta and EPCR from the transfected cell line (E7) exhibited similar mobility on SDS-PAGE. The apparent mass of the EPCR (40 kDa) on SDS-PAGE is approximately twice that predicted from the amino acid sequence, consistent with the predicted four N-linked carbohydrate attachment sites on EPCR (5). Some degradation in the placental EPCR was often observed. Considerably less degradation was observed in the EPCR isolated from the E7 cells.

Mono Q chromatography of the immunoaffinity-purified TM resulted in a major peak with a shoulder on the trailing edge. This shoulder was less than 30% of the TM preparation (data not shown). TM isolated in the major peak from the Mono Q column (i.e. largely devoid of chondroitin sulfate, see below) is shown in Fig. 1B. As has been observed with other human TM preparations from placenta (25), there are aggregates and some degradation products present in addition to the major monomeric TM form. Most of these aggregates could be dissociated by disulfide bond reduction, consistent with the known Cys residues in the cytoplasmic tail (26–28). The presence of a trailing shoulder on the main peak from the Mono Q column behavior is indicative of relatively anionic forms of human TM. This anionic character appears to be caused by modification with chondroitin sulfate. Western blots of the trailing fractions revealed a diffuse band that was sharpened after chondroitinase ABC treatment (Fig. 1C). Rabbit TM has been shown to contain a chondroitin sulfate moiety that renders the molecule more anionic, and the proteoglycan form migrates as a diffuse, slower moving band when analyzed by SDS-PAGE (29). Preliminary data with human TM preparations are also consistent with this proposal (30).

Antigen levels of TM and EPCR in the Triton X-100 extract of the placenta were determined by ELISA. Assuming nearly complete extraction, there were approximately 0.22 mg of EPCR and 0.88 mg of TM per placenta. The higher TM:EPCR ratio is consistent with the observation that EPCR is found mostly in large vessels, and TM is in both large and microvessels, the latter constituting the vast majority of the endothelium (8).
The availability of purified human TM and EPCR allowed us to reconstitute the isolated proteins into membrane vesicles using the detergent dialysis technique (22, 31). Proteins incorporated into the phospholipid can be separated from free protein by sucrose density gradient centrifugation in which the liposomes are found at the top of the gradient and the free protein near the bottom. As can be seen in Fig. 2, placental TM and EPCR from either tissue culture or from placenta can be incorporated into vesicles in this fashion. It should be noted that EPCR at either low or high concentrations does not incorporate as efficiently as TM. This is possibly because of the presence of two Gly-Gly sequences in the transmembrane region (5) which would tend to break helix formation at the beginning and end of the transmembrane region. Placental and recombinant EPCR were indistinguishable with respect to phospholipid reconstitution and functional properties and are used interchangeably in the following experiments. When the EPCR that did not incorporate into phospholipid was isolated and the reconstitution protocol was repeated, the EPCR again failed to incorporate into the liposomes (data not shown). The molecular basis for this behavior is currently under investigation.

The EPCR concentration dependence of protein C activation was examined by adjusting the EPCR concentration during the reconstitution. It was possible to vary the EPCR:TM ratio from 1:1 to 14.2:1. As the EPCR ratio increased, the rate of protein C activation increased (Fig. 3). To separate the EPCR effects on the activation reactions from variations in TM incorporation, we took two separate approaches. First, we determined the activation rate of rGDPC. Without the Gla domain, protein C can be activated by the soluble thrombin-TM complex, but the activation rate is not influenced by reconstitution into neutral or negatively charged phospholipid (22), and the molecule cannot bind to EPCR (5). Second, activation rates in the presence of anti-EPCR mAb 1494 which blocks protein C binding to the EPCR on cell surfaces (6). When the rate of activation as a function of protein C concentration was examined (Fig. 4), it was apparent that the affinity for protein C was increased by either EPCR or the presence of negatively charged phospholipids. Fitting the data to Michaelis-Menten kinetics indicated that on phosphatidylcholine-containing vesicles, the $K_m$ was $2.17 \pm 0.13 \mu M$ without EPCR. With EPCR present, the data could not be fit well to a two-site model with a tight site, $K_m = 20 \pm 15 \mu M$, and a weak site, $K_m = 3.3 \pm 1.7 \mu M$ (Fig. 5B). Increasing the EPCR:TM ratios from 8:6:1 to 14:1 increased the percentage of tight sites from 14 to 23%. The necessity for a two-site model to fit the data was only observed with the EPCR-containing liposomes. Michaelis-

**FIG. 2.** SDS-PAGE analysis of EPCR and TM incorporation into liposomes. Fractions from a sucrose density gradient separating free and phospholipid incorporated EPCR and TM were subjected to 10% SDS-PAGE. Left panel, human placental EPCR and TM incorporation into phosphatidylcholine vesicles with an EPCR:TM ratio of 0.6 (w/w) in the reconstitution mixture; right panel, EPCR isolated from E7 cells and placental TM were reconstituted into phosphatidylcholine vesicles at a 5:6:1 w/w ratio.

**FIG. 3.** Influences of EPCR concentration or negatively charged phospholipids on protein C and rGDPC activation rates. EPCR and TM were reconstituted into phosphatidylcholine vesicles (TM/PC) at the EPCR:TM ratios indicated. Gray bars, activation rate of rGDPC; black bars, activation rate of protein C; white bars, activation rate of protein C in the presence of 0.1 µM inhibitory anti-EPCR mAb 1494. TM was also incorporated into vesicles containing 20% phosphatidylserine, 80% phosphatidylcholine (TM-PS/PC), 40% phosphatidylethanolamine, 60% phosphatidylcholine (TM-PE/PC), or 20% phosphatidylethanolamine, 40% phosphatidylethanolamine, 40% phosphatidylcholine (TM-PS/PE/PC). Gray bars, activation rate of rGDPC; black bars, activation rate of protein C; hatched bars, activation rate of protein C in the presence of 1 µM annexin V. TM concentrations on these liposomes were (from left to right) 0.078, 0.124, 0.28, 0.444, 0.142, 0.172, and 0.218 nM. EPCR surface concentrations were 1.11, 1.07, and 0.28 nM. The phospholipid concentration was 20 µg/ml in all cases. The initial rates of protein C activation were performed at 37°C as described under “Experimental Procedures.” All assays were performed in duplicate, and all errors were within 5%. Reducing protein C activation rates primarily by increasing the $K_m$ (6). When the rate of activation as a function of protein C concentration was examined (Fig. 4), it was apparent that the affinity for protein C was increased by either EPCR or the presence of negatively charged phospholipids. Fitting the data to Michaelis-Menten kinetics indicated that on phosphatidylcholine-containing vesicles, the $K_m$ was $2.17 \pm 0.13 \mu M$ without EPCR. With EPCR present, the data could not be fit well to a two-site model with a tight site, $K_m = 20 \pm 15 \mu M$, and a weak site, $K_m = 3.3 \pm 1.7 \mu M$ (Fig. 5B). Increasing the EPCR:TM ratios from 8:6:1 to 14:1 increased the percentage of tight sites from 14 to 23%. The necessity for a two-site model to fit the data was only observed with the EPCR-containing liposomes. Michaelis-

Incorporation of TM into vesicles with or without EPCR had (Fig. 3) but not by the anti-EPCR mAb 1494 (data not shown). Annexin V served with rGDPC (Fig. 3), but annexin V had no influence in this system (data not shown). Inclusion of phosphatidylethanolamine, or a combination of the phospholipids in the vesicles accelerated protein C activation in the absence of EPCR. This acceleration was inhibited by annexin V (Fig. 3) but not by the anti-EPCR mAb 1494 (data not shown). Incorporation of TM into vesicles with or without EPCR had little if any effect on the affinity of TM for thrombin ($K_m^{app} \approx 2.5 \mu M$), consistent with previous studies that failed to demonstrate a phospholipid influence on the affinity of thrombin for TM (7, 22).

Blocking protein C binding to the EPCR on cell surfaces reduces protein C activation rates primarily by increasing the $K_m$ (6). When the rate of activation as a function of protein C concentration was examined (Fig. 4), it was apparent that the affinity for protein C was increased by either EPCR or the presence of negatively charged phospholipids. Fitting the data to Michaelis-Menten kinetics indicated that on phosphatidylcholine-containing vesicles, the $K_m$ was $2.17 \pm 0.13 \mu M$ without EPCR. With EPCR present, the data could not be fit well to a two-site model with a tight site, $K_m = 20 \pm 15 \mu M$, and a weak site, $K_m = 3.3 \pm 1.7 \mu M$ (Fig. 5B). Increasing the EPCR:TM ratios from 8:6:1 to 14:1 increased the percentage of tight sites from 14 to 23%. The necessity for a two-site model to fit the data was only observed with the EPCR-containing liposomes. Michaelis-

**FIG. 3.** Influences of EPCR concentration or negatively charged phospholipids on protein C and rGDPC activation rates. EPCR and TM were reconstituted into phosphatidylcholine vesicles (TM/PC) at the EPCR:TM ratios indicated. Gray bars, activation rate of rGDPC; black bars, activation rate of protein C; white bars, activation rate of protein C in the presence of 0.1 µM inhibitory anti-EPCR mAb 1494. TM was also incorporated into vesicles containing 20% phosphatidylserine, 80% phosphatidylcholine (TM-PS/PC), 40% phosphatidylethanolamine, 60% phosphatidylcholine (TM-PE/PC), or 20% phosphatidylethanolamine, 40% phosphatidylethanolamine, 40% phosphatidylcholine (TM-PS/PE/PC). Gray bars, activation rate of rGDPC; black bars, activation rate of protein C; hatched bars, activation rate of protein C in the presence of 1 µM annexin V. TM concentrations on these liposomes were (from left to right) 0.078, 0.124, 0.28, 0.444, 0.142, 0.172, and 0.218 nM. EPCR surface concentrations were 1.11, 1.07, and 0.28 nM. The phospholipid concentration was 20 µg/ml in all cases. The initial rates of protein C activation were performed at 37°C as described under “Experimental Procedures.” All assays were performed in duplicate, and all errors were within 5%.
Menten kinetics fit the data well even when liposomes contained TM, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (Fig. 5C) ($K_m = 0.89 \pm 0.22$ nM). The $K_{cat}$ values for all reaction mixtures were similar. For phosphatidylcholine liposomes containing EPCR and TM (ratio = 14:2:1), the $K_{cat}$ was $11.1 \pm 1.7$ mol of APC/min/mol of TM. For liposomes containing TM, phosphatidylethanolamine, and phosphatidylcholine, the $K_{cat}$ was $8.0 \pm 0.2$ mol of APC/min/mol of TM. The $K_{cat}$ values observed here are much lower than those observed with rabbit TM, about 250 mol/min/mol of TM (32), but are comparable to human placental TM reconstituted into negatively charged vesicles (8 mol/min/mol of TM) (7) reported previously.

To ascertain whether the absolute density of the EPCR and TM on the liposome surface plays an important role in EPCR acceleration, we varied the amount of both the EPCR and TM incorporated into the liposomes by changing the phospholipid: protein ratio during the reconstitution. The ratios of protein C to rGDPc activation rates were then used as a monitor of EPCR function. As the density of the receptors on the vesicle surface decreased (Fig. 6, highest density on the left, lowest on the right), the selective enhancement of protein C activation over rGDPc activation decreased progressively. The selective enhancement of protein C over rGDPc activation rates was eliminated by the anti-EPCR mAb 1494 (hatched bars), indicating that the loss of this property was a result of the loss of EPCR function and not a property of altered TM:phospholipid ratios.

To determine the relationship between the reconstituted system and activation over HUVEC, we estimated the TM and EPCR concentrations on the cells using $^{125}$I-radiolabeled mAb Fab fragments to EPCR and TM. When binding was performed as described under “Experimental Procedures,” we estimated about 700,000 EPCR molecules and 100,000 TM molecules/ HUVEC. This estimate of surface TM expression on HUVEC is slightly higher than the earlier estimate of 30,000–55,000 based on intact mAb binding (33). Blocking protein C binding to EPCR with anti-EPCR mAb 1494 decreased the activation rate on HUVEC 9-fold (Fig. 7). In contrast, annexin V, which effectively inhibits protein C activation on negatively charged vesicles (Fig. 3), had little influence on protein C activation over quiescent HUVEC. Similar rates of protein C activation were observed over HUVEC (2.7 mol of APC/min/mol of TM) and phosphatidylcholine vesicles with an 8.6:1 EPCR:TM ratio (3.2 mol of APC/min/mol of TM). Thus, the contribution of the HUVEC surface to protein C activation can be adequately accounted for with the simple system containing phosphatidylcholine, EPCR, and TM. Negatively charged phospholipids appear to contribute little to this process.

FIG. 4. Influence of EPCR and negatively charged phospholipid on protein C activation as a function of protein C concentrations. Phosphatidylcholine vesicles containing TM and EPCR (2) had an EPCR:TM ratio of 8.6:1, 0.124 nM TM and 20 μg/ml phosphatidylcholine in the activation mixture. The TM and phosphatidylcholine vesicles devoid of EPCR (3) had a TM concentration of 0.44 nM and phosphatidylcholine concentration of 20 μg/ml in the activation mixture. The 20% phosphatidylserine, 40% phosphatidylethanolamine, 40% phosphatidylcholine liposomes containing TM (●) had a TM concentration of 0.218 nM and a phospholipid concentration of 20 μg/ml in the activation mixture. The initial rates of protein C activation were determined at the protein C concentrations indicated on the x axis. The inset expands the graph to focus on activation rates at low protein C concentrations.

FIG. 5. Analysis of the protein C concentration dependence of activation. Panel A, the rate of protein C activation on phosphatidylcholine vesicles containing a 14:1 EPCR:TM ratio (TM = 0.078 nM) was examined as a function of increasing substrate concentration, and the data were fit to Michaelis-Menten kinetics using the Ultrafit program for Macintosh from Biosoft. Panel B, the data from panel A were fit to a two-site model where $v = (V_{maxA}(K_{m(A)} + C) + V_{maxB}(K_{m(B)} + C)$ where $v$ is the observed velocity, $V_{maxA}$ is the $V_{max}$ of the high affinity sites, $V_{maxB}$ is the $V_{max}$ of the low affinity sites, $C$ is the concentration of protein C, $K_{m(A)}$ is the $K_m$ of the high affinity site, and $K_{m(B)}$ is the $K_m$ of the low affinity sites. The data were fit using the Ultrafit program. Panel C, the rate of protein C activation on vesicles containing 20% phosphatidylserine, 40% phosphatidylethanolamine, 40% phosphatidylcholine, and 0.218 nM TM was studied as a function of protein C concentration, and the data were fit to Michaelis-Menten kinetics using the Ultrafit program.
FIG. 6. Influence of EPCR and TM surface density on the protein C activation. TM (5, 2.5, 1.25, and 0.625 μg) was reconstituted into 1 mg of phosphatidylcholine-containing vesicles with corresponding decreasing amounts of EPCR (13.7, 6.85, 3.43, and 1.72 μg). The mixtures were brought to 200 μl and the reconstituted liposomes isolated and characterized as described under “Experimental Procedures.” The concentrations of phospholipid ([PL]) and TM are indicated on the figure, as are the EPCR:TM ratios of each sample. The ratio of the rate of protein C activation to the rate of rGDPC activation was taken as a measure of EPCR effectiveness. These assays were performed in the presence (hatched bars) and absence (solid bars) of the inhibitory anti-EPCR mAb 1494 (0.1 μM).

FIG. 7. Protein C activation by HUVEC is effectively inhibited by an anti-EPCR mAb but not by annexin V. Protein C or rGDPC activation by HUVEC was measured as described under “Experimental Procedures.” rGDPC activation rates were similar to the activation rate of protein C in the presence of 0.1 μm mAb 1494 (anti-EPCR + PC) and much slower than protein C in the absence of the antibody (PC). Recombinant annexin V (1 μm) was not an effective inhibitor of protein C activation (rAn V + PC). Inhibiting thrombin binding to TM with anti-TM mAb CTM1009 (0.1 μM) almost eliminated protein C activation (anti-TM + PC). The TM concentrations on the cell surface were estimated using 125I-Fab fragments and corresponded to approximately 100,000 sites/endothelial cell. All determinations were performed in duplicate.

**DISCUSSION**

Previous studies demonstrated that inhibition of EPCR-protein C interaction could reduce protein C activation rates (6) and that coexpression of EPCR and TM increased the TM-dependent protein C activation rate (10). Both of these studies demonstrate involvement of EPCR in protein C activation, but neither rules out a requirement for another cellular component in this process. The present study demonstrates that EPCR and TM are the only components required to enhance protein C activation. The protein C activation rates by HUVEC with EPCR:TM ratios of approximately 7:1 were similar to those obtained with EPCR and TM reconstituted into phosphatidylcholine vesicles at ratios of 8:6:1, i.e. 2.7 versus 3.2 mol/min/mol of TM-thrombin complex. In addition, the rates of protein C activation compared with rGDPC activation were about 9-fold greater on both endothelium and phosphatidylcholine liposomes containing an EPCR:TM ratio of 8:6:1. The ability to inhibit protein C activation over quiescent HUVEC with the anti-EPCR mAb, but not with annexin V, argues strongly that the protein C interaction with the cell surface that augments activation is mediated primarily if not exclusively by EPCR rather than by negatively charged phospholipids. This provides selectivity for protein C activation because the other vitamin K-dependent proteins would compete effectively for the phospholipid surface and inhibit protein C activation, but these factors do not compete effectively for binding to EPCR (34).

The enhancement observed with EPCR is small compared with other coagulation cofactors that can increase activation rates thousands of fold (35). One might question whether this relatively small change in activation rate is biologically important. The answer would appear to be yes because it is now well established that even a 2-fold reduction in protein C concentration can result in an increased risk of venous thrombosis (36). This propensity toward thrombosis is almost certainly the result of a reduction in protein C activation rates associated with the decrease in circulating protein C concentration rather than consumption because very little protein C is activated tonically (37). Therefore, increases in protein C activation rates of 9-fold or greater as observed here are likely to play an important role in regulating the blood coagulation process in large vessels. The much higher effective TM concentration in the microcirculation can probably generate sufficient APC to prevent microvascular thrombosis as long as the protein C concentration remains high. Consistent with this hypothesis, partial reductions of protein C concentration are usually associated with large vessel thrombosis, and very low levels are associated with microvascular thrombosis (36, 38).

Immunohistochemical studies revealed previously that EPCR expression is much greater on large vessel endothelium, particularly large arteries, and decreases until it is nearly undetectable in most capillary endothelial beds (9). The observation that protein C activation rates rise with increasing concentrations of EPCR provides the first evidence that there is an EPCR concentration dependence to protein C activation even when the EPCR concentration exceeds that of TM. The most likely explanation for the EPCR concentration dependence (lack of apparent saturation) observed in this study is that there is little or no direct interaction between TM and EPCR. Otherwise, one would predict saturation at low integer ratios of
EPCR to TM, especially given that the local concentrations expressed in two dimensions are quite high for these reconstituted proteins. The conclusion that there is a lack of appreciable affinity between EPCR and TM is bolstered by the observation that the phospholipid to protein ratio increases, thereby decreasing the surface density of EPCR and TM, the efficiency of the thrombin-TM complexes is decreased. This is true even though the EPCR to TM ratios remained essentially constant (Fig. 6).

Analysis of the protein C concentration dependence over liposomes containing EPCR and TM revealed a complex pattern that did not fit simple Michaelis-Menten kinetics, whereas activation over liposomes devoid of EPCR fit Michaelis-Menten kinetics very well. The curve with EPCR-containing vesicles was consistent with a population of activation complexes that were high affinity for protein C and another population that was low affinity (see Figs. 4 and 5). The high affinity sites ($K_a \approx 20$ nM) are considerably below the plasma protein C concentration, whereas the low affinity sites ($K_a \approx 2-3$ nM) are considerably above this value. The high affinity $K_a$ is similar to the $K_d$ for protein C/APC binding to EPCR estimated in previous studies (30–50 nM) (5). One interpretation of the apparent two classes of sites is that the higher affinity sites involve the EPCR-protein C complex, but the low affinity sites represent catalysis by the free thrombin-TM complex. Further analysis of the complex kinetic pattern was not attempted because it would require knowledge of lateral mobility and the off rate of the APC from EPCR.

The present study demonstrates that the EPCR concentration plays a major role in determining protein C activation. Up-regulation of EPCR by thrombin (12) or down-regulation of EPCR expression by cytokines (50) or proteolytic attack (39, 40) would, based on the present study, contribute directly to protein C activation and hence serve to modulate the critical control of the blood clotting process. 

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