Enhancement of thrombin–thrombomodulin-catalysed protein C activation by phosphatidylethanolamine containing unsaturated fatty acids: possible physiological significance of phosphatidylethanolamine in anticoagulant activity of thrombomodulin

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The effects of phospholipid vesicles and their fatty acid compositions on the acceleration of Protein C activation by thrombin–thrombomodulin was studied *in vitro*. Four main phospholipid fractions were prepared from cultured human umbilical vein endothelial cells, and purified thrombomodulin from human placenta was reconstituted into vesicles consisting of phosphatidylcholine (PtdCho) alone, PtdCho plus phosphatidylethanolamine (PtdEtn), PtdCho plus phosphatidylserine (PtdSer) and PtdCho plus PtdIns (1:1, w/w in each case). Vesicles of PtdCho, PtdIns/PtdCho, PtdSer/PtdCho and PtdEtn/PtdCho increased thrombin–thrombomodulin-catalysed protein C activation by 1.2-, 1.9-, 4.3- and 8.4-fold respectively compared with that in the absence of phospholipid. This Protein C activation was not affected by distearoyl PtdEtn/distearoyl PtdCho, whereas it was markedly increased with increasing content of unsaturated fatty acid in PtdEtn. The thrombin-dependent Protein C activation by thrombomodulin re-

constituted into dilinolenoyl PtdEtn/distearoyl PtdCho was 14.6 times that by thrombomodulin reconstituted into distearoyl PtdEtn/distearoyl PtdCho, as a result of a decrease in the dissociation constant (K_d) for thrombin and the Michaelis constant (K_m) for Protein C of thrombomodulin. Binding of Protein C to PtdEtn/PtdCho fixed to a microwell plate required the presence of CaCl_2 and increased with increasing degree of unsaturation of fatty acid in PtdEtn. As PtdEtn appeared on the outside of the plasma membrane in cultured human umbilical vein endothelial cells after thrombin stimulation, it was presumed that Protein C activation could be elevated by PtdEtn at the outer surface of the plasma membrane via an increased affinity between thrombomodulin, thrombin and Protein C, resulting from both increased formation of the thrombin–thrombomodulin complex via a conformational change in thrombomodulin and increased binding of Protein C to the membrane phospholipid in a Ca^{2+} -dependent manner.

INTRODUCTION

Endothelial cells play an important role in the control of thrombosis and haemostasis on the membrane. Protein C, a vitamin K-dependent zymogen, is converted into its active form by thrombin and the activated Protein C serves as an anticoagulant by inactivation of Factors Va and VIIIa by limited proteolytic cleavage [1–3]. Thrombomodulin, a glycoprotein on the endothelial cell surface, is a high-affinity receptor for thrombin, and the thrombin–thrombomodulin complex shows potent anticoagulant properties through marked acceleration of Protein C activation compared with that produced by thrombin alone [4–7]. Expression of thrombomodulin on the surface of endothelial cells is increased by treatment with dibutyryl cyclic AMP [8,9] or retinoic acid [10,11], and is decreased by exposure to tumour necrosis factor α [11–13] or interleukin 1β [9].

Thrombin- and Protein C-binding sites of thrombomodulin have been examined and it has been shown that thrombin and Protein C bind to the fifth [14] and fourth [15,16] epidermal growth factor-like structures respectively of thrombomodulin. Preissner et al. [17] suggested that a secondary thrombin-binding site possibly exists on the acidic domain of thrombomodulin, a sulphated glycosaminoglycan. However, no particular attention

was paid in these studies to the influence of the phospholipid-rich environment of the endothelial cell surface.

In 1982, Esmon et al. [18] purified thrombomodulin from rabbit lung and first described the apparent inconsistency between its cofactor activity and its activity on endothelial cells. They suggested that the decrease in thrombin-dependent Protein C activation by thrombomodulin might be due to loss of phospholipids during the purification procedure [18]. Freyssinet et al. [19] reconstituted thrombomodulin into phospholipid vesicles, and suggested that anionic phospholipids might be of functional significance in the activation of Protein C by the thrombin–thrombomodulin complex. They proposed that the positive effect involved a Ca^{2+} -dependent interaction between Protein C and phospholipids. On the other hand, Galvin et al. [20] suggested that the increase in Protein C activation caused by reconstituted thrombomodulin might involve a Ca^{2+} -dependent interaction between Protein C and thrombomodulin, which exposes a Protein C-binding site after conformational change that results from its interaction with phospholipids. Thus Protein C activation on endothelial-cell membrane is controlled not only by the amount of thrombomodulin expressed but also by changes in the membrane environment, such as phospholipid composition. In order to clarify further the physiological control mechanism of

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; 18:0–18:0, distearoyl; 18:1–18:1, dioleoyl; 18:2–18:2, dilinoleoyl; 18:3–18:3, dilinolenoyl; Gla, γ -carboxyglutamic acid; Boc, *t*-butoxycarbonyl; MCA, 4-methylcoumarin-7-amide; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; ECGS, endothelial growth supplement; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

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thrombin-dependent Protein C activation by thrombomodulin (cofactor activity of thrombomodulin) on the endothelial cell surface, which is rich in phospholipids containing unsaturated fatty acid [21], we investigated the effect of the composition of phospholipid vesicles on this cofactor activity.

In the present study, we reconstituted human placental thrombomodulin into phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) vesicles, which exhibited the largest increase in thrombin–thrombomodulin-catalysed Protein C activation among various phospholipid vesicles tested, and we investigated the effect of unsaturated fatty acid in PtdEtn/PtdCho vesicles on the cofactor activity of thrombomodulin and on the interactions between thrombomodulin, thrombin and Protein C. It is proposed that a loss of preferential distribution of PtdEtn on the inside of the endothelial plasma membrane and its appearance on the outside of the membrane may modulate the cofactor activity of thrombomodulin.

EXPERIMENTAL

Materials

All phospholipids were purchased from Avanti Polar Lipids Inc., Alabaster, AL, U.S.A. Human Protein C was obtained from American Diagnostica Inc., Greenwich, CT, U.S.A. Thrombin was donated by the Green Cross, Osaka, Japan, and was further purified by benzamidine–Sephacryl column chromatography. Chymotrypsin, heparin, BSA (fatty acid-free) and *o*-phenylenediamine were obtained from Sigma, St. Louis, MO, U.S.A. Boc-Leu-Ser-Thr-Arg-MCA and 7-amino-4-methylcoumarin were purchased from Peptide Institute, Osaka, Japan. Dulbecco's minimal essential medium (DMEM) and fetal calf serum (FCS) were purchased from Flow Laboratories, Irvine, Scotland, U.K., and Hyclone Laboratories, Logan, UT, U.S.A. respectively. Chondroitin ABC lyase was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Collagen-coated dishes (60 and 100 mm diameter) and 175 cm² tissue culture flasks were purchased from Corning–Iwaki Glass, Tokyo, Japan. Uncoated dishes and microwell plates were purchased from Falcon, Becton Dickinson and Company, Lincoln Park, NJ, U.S.A. and Japan Inter Med Co., Tokyo, Japan respectively. Endothelial growth supplement (ECGS) and trypsin were obtained from Collaborative Research, Waltham, MA, U.S.A., and DIFCO Laboratories, Detroit, MI, U.S.A. respectively. Resins for column chromatography were obtained from Pharmacia Co., Tokyo, Japan. Other reagents, unless otherwise indicated, were purchased from Wako Pure Chemicals, Osaka, Japan. Activated Protein C was donated by Eli Lilly Co., Indianapolis, IN, U.S.A. Mouse monoclonal anti-(human thrombomodulin) IgGs (TMmAbs 2, 11 and 20) were prepared and labelled with horseradish peroxidase (HRP) as reported previously [22]. Mouse monoclonal anti-(human Protein C) IgG (PCmAb 7) which binds to the heavy chain of Protein C, recognizing the conformational structure of Protein C, was prepared in the same way as the TMmAbs.

Purification of thrombomodulin from human placenta

Thrombomodulin was purified from human placenta as described by Salem et al. [23] with some modifications. Briefly, thrombomodulin extracted from the tissue by treatment with 0.5% Lubrol PX was subjected to column chromatography on DEAE-cellulose, di-isopropylphospho-thrombin agarose and polyclonal antibody-coupled Sepharose 4B. Protein purity was examined by SDS/PAGE followed by staining with Coomassie Brilliant Blue. The purified thrombomodulin prepared from human placenta

had a molecular mass of 105 kDa and was more than 90% pure as evaluated with a flying spot scanner SC-6000 (Shimadzu Co., Tokyo, Japan).

Recombinant thrombomodulin

Recombinant (r-) thrombomodulin was a gift from the Research Institute, Daiichi Pharmaceutical Co. It was expressed by introduction of pRS7TM into CHO-K1 cells, and the secreted thrombomodulin was purified from the culture medium by chromatography on Q-Sepharose Fast Flow (Pharmacia-LKB) and anti-thrombomodulin monoclonal antibody Cellulofine followed by Sephacryl S-300 columns as described by Nawa et al. [24]. This r-thrombomodulin contains 491 amino acids from N-terminal Ala-1 to C-terminal Ala-491, and lacks the transmembrane and cytoplasmic domains of endogenous thrombomodulin. All of the potential N- and O-glycosylation sites were the same as those of native thrombomodulin.

Culture of human endothelial cells

Endothelial cells were isolated from human umbilical cord veins by the method of Jaffe et al. [25], and were cultured in 60 or 100 mm-diameter collagen-coated dishes containing culture medium consisting of DMEM supplemented with 20% (v/v) FCS, 20 µg of ECGS/ml, 100 units of heparin/ml and antibiotics (50 µg of streptomycin/ml, 50 µg of penicillin/ml) [10]. Confluent primary cultures were passaged and cultured under the same conditions to confluency and then seeded in collagen-coated or uncoated dishes. These cells were grown to confluence under 5% CO₂/95% O₂.

Extraction of phospholipids from cultured endothelial cells

Phospholipids were extracted from cultured human umbilical vein endothelial cells (HUVECs). Approximately 400 µg of total phospholipids was extracted from HUVECs harvested on 100 mm-diameter collagen-coated dishes (approx. 2.5 × 10⁶ cells/dish); the PtdEtn separated from one dish was sufficient to provide at least two assay samples for evaluating cofactor activity of thrombomodulin. Large-scale (in 175 cm² flasks) secondary or tertiary cultures were washed three times with PBS and the cells were collected with a rubber 'policeman'. In total, 3 × 10⁷ cells from six flasks were used as the starting material. The cell suspensions were homogenized with a Potter–Elvehjem-type homogenizer (80 strokes) and centrifuged at 600 g for 10 min. The supernatant was centrifuged at 10 500 g for 15 min and the resulting supernatant was further centrifuged for 1 h at 105 000 g. The precipitated membrane-rich fraction was suspended in PBS and total lipids were extracted with chloroform/methanol (1:2, v/v). The insoluble fraction was discarded by centrifugation, then 0.15 M NaCl was added and the resulting chloroform layer was dried under a nitrogen stream [26]. The residue was spotted on silica gel 60F-254 (Merck) and developed first with chloroform/methanol/ammonium hydroxide (65:25:4, by vol.), followed by chloroform/methanol/ammonium hydroxide (50:25:6, by vol.) [27]. For the preparation of both PtdEtn and PtdCho, only the second development was performed. The identity of each phospholipid fraction was confirmed by comparison with authentic standards after spraying with primulin, and the appropriate region of the plate was scraped off and extracted with chloroform/methanol (2:1, v/v). The concentration of phospholipid was determined by measuring inorganic phosphorus by the method of Bartlett [28], using H₂SO₄ for digestion.

Reconstitution of thrombomodulin into phospholipid vesicles

The PtdCho and/or a PtdCho-individual phospholipid mixture (1:1, w/w) in chloroform were dried under a nitrogen stream and phospholipid vesicles were prepared in a buffer system (buffer A) containing 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 5 mg/ml BSA, by the method of Huang and Thompson [29] with slight modifications. The phospholipid suspensions were sonicated three times for 5 min each using a Sonifier (Branson Sonic Power Co., model W-200P, Danbury, CT, U.S.A.), instead of 2 h, to obtain larger vesicles [30]. In order to avoid oxidation of the phospholipids, the method described by Freyssinet et al. [30] was used to reconstitute thrombomodulin in all the present experiments. Briefly, purified thrombomodulin (0.4 μ M in 0.1 % Lubrol PX, 20 mM Tris/HCl, pH 7.5, and 0.15 M NaCl) was added to the sonicated phospholipid suspensions and then incubated for more than 30 min at 20–25 °C. After this procedure, the concentration of Lubrol PX in the reaction mixture became 0.0005 %, which was below the critical micelle concentration. The molar ratio of phospholipid to thrombomodulin was 316500:1 at a final concentration of 0.5 mg of phospholipid/ml (calculated on the basis of an M_r of 790 for distearoyl PtdCho). As reported by Freyssinet et al. [30], approximately one-half of thrombomodulin molecules must be localized on the inner side of the vesicles, because double the cofactor activity of thrombomodulin was found in the vesicles after treatment of the vesicles with 1 % Triton X-100 (final concentration). Rabbit thrombomodulin was reconstituted into phospholipid vesicles by the same method.

Sucrose-density-gradient centrifugation and measurement of thrombomodulin antigen

Thrombomodulin reconstituted into phospholipid vesicles (150 μ l; phospholipid-to-thrombomodulin molar ratio 316500:1) was layered on a discontinuous sucrose gradient [30, 25, 20, 15, 10, 5 % (w/w) in 0.15 M NaCl/20 mM Tris/HCl, pH 7.5, 2.5 ml each in order from the bottom] and centrifuged in a Hitachi swinging rotor (RPS-28-A1) for 16 h at 120000 *g*. Fractions of 1 ml each were obtained from the bottom with a peristaltic pump and then the amount of thrombomodulin antigen and the phospholipid content were measured. The total thrombomodulin antigen level was determined by using horseradish peroxidase (HRP)-labelled monoclonal antibodies against thrombomodulin (TMmAb2 and 11) as described previously [22] after treatment of fractions with 1 % Triton X-100 for 30 min at room temperature. More than 80 % of thrombomodulin was recovered in the 0–15 % sucrose fraction of the gradient when thrombomodulin was reconstituted into phospholipid vesicles, whereas less than 2 % was recovered in that fraction in the absence of phospholipid.

Determination of cofactor activity of thrombomodulin

The ability of thrombomodulin to accelerate thrombin-dependent Protein C activation was determined by the method of Tsiang et al. [31] with slight modifications. In short, thrombomodulin reconstituted into phospholipid vesicles was incubated in a medium containing 20 nM thrombin, 2 mM CaCl_2 , 2 μ M human Protein C/ml, 5 mg of BSA/ml and 0.15 M NaCl in 20 mM Tris/HCl (pH 7.4). Incubation was performed at 37 °C for 30 min and then thrombin was inactivated by the addition of a mixture of antithrombin III (final concentration 2 units/ml) and heparin (final concentration 8 units/ml). Boc-Leu-Ser-Thr-Arg-MCA, a synthetic substrate of activated Protein C, was added to the mixture and incubated for 10 min at 37 °C. After the addition of acetic acid, 7-amino-4-methylcoumarin liberated

was measured by using a Hitachi spectrofluorimeter with excitation at 380 nm and emission at 440 nm, and the amount of activated Protein C was calculated from the activity of pure activated Protein C.

Preparation of γ -glutamic acid (Gla)-domainless Protein C

Gla-domainless Protein C was prepared by incubation of Protein C with chymotrypsin (at an enzyme/Protein C ratio of 1:400, w/w) for 30 min at 37 °C in 20 mM Tris/HCl (pH 7.4) containing 0.1 M NaCl [32]. After inactivation of the enzyme with diisopropyl fluorophosphate, the mixture was applied to a QAE-Sephadex A-50 column as described by Esmon et al. [32]. The protein fraction eluted with an NaCl gradient was dialysed and concentrated with a Centricon-30 membrane (Amicon). The purity of Gla-domainless Protein C in each fraction was determined by gel electrophoresis on 10 % acrylamide. The purity of the final sample was more than 92 % in terms of the intensity of Coomassie Brilliant Blue staining.

Binding assays of thrombomodulin and Protein C to phospholipids

To evaluate binding of thrombomodulin and Protein C to phospholipids, PtdCho or PtdCho/PtdEtn vesicles (1 mg/ml of buffer A) prepared as above were coated on to microwell plates (96 wells) and allowed to stand for 4 h at 25 °C. Unbound phospholipids were removed and wells were washed three times at 30 min intervals with buffer B consisting of 50 mM Hepes (pH 7.5), 0.15 M NaCl and 1 % (w/v) BSA. When the actual amount of each phospholipid fixed on the well was determined, the equivalent of approx. 12 μ g/well was obtained irrespective of the phospholipid composition. Purified thrombomodulin (1–10 nM) or Protein C (50–400 nM) in 20 mM Tris/HCl (pH 7.5) containing 0.15 M NaCl and 10 mM benzamidine hydrochloride was added to the wells in the presence or absence of 2 mM CaCl_2 and the plate was allowed to stand for 2 h at 25 °C. After three washes with buffer B, a 200-fold excess of monoclonal antibody with respect to the concentration of thrombomodulin or Protein C was added and the mixture was left to stand for 1 h at 25 °C. After three washes, the amount of thrombomodulin or Protein C bound to phospholipid was measured by determination of the HRP activity of the appropriate HRP-labelled mouse monoclonal IgG bound to each protein.

Incubation of endothelial cells with 2,4,6-trinitrobenzenesulphonic acid (TNBS)

In order to determine the location of PtdEtn in HUVEC membrane, the intact cells were allowed to react with TNBS, an amino-group-modifying reagent [33,34], under non-permeable conditions as described by Olaisson et al. [26] with a slight modification. HUVECs in 60 mm-diameter culture dishes were washed twice with PBS, then 10 mM disodium phosphate buffer, pH 7.9, containing 0.25 M sucrose was added. In the presence of 0.2 mM CaCl_2 (final concentration), thrombin (0.5 NIH unit/ml final concentration) was added to the culture. After 20 min, 40 mM TNBS (final) in 10 mM disodium phosphate buffer (pH 7.9) containing 0.25 M sucrose and 0.2 mM CaCl_2 was added. For the control the same buffer without thrombin was used. Incubation was carried out at 37 °C for 40 min in a final volume of 2 ml and the reaction was terminated by the addition of 400 μ l of 0.2 M acetic acid. The cells were collected with a rubber 'policeman' and then transferred to test tubes with washing. After the addition of chloroform and methanol, the

tube was vigorously shaken and left to stand for 1 h. The insoluble fraction was removed by centrifugation and each sample was separated into two phases by the addition of 0.15 M NaCl, and then neutralized with NaOH. After centrifugation, the lower phase was recovered and dried in a nitrogen stream. The residue was dissolved in chloroform/methanol (2:1, v/v) and separated on a silica gel 60F-254 plate, using a mobile phase consisting of chloroform/methanol/ammonia (50:25:6, by vol.). The lipid spot visualized in yellow (trinitrophenylated PtdEtn) and the PtdEtn spot detected with primulin spray were scraped off and phospholipid phosphorus was determined as described above.

RESULTS

Phospholipid composition in endothelial cells and the effect of phospholipid classes on the cofactor activity of thrombomodulin for thrombin-dependent Protein C activation

In order to understand the physiological significance of phospholipid classes on the cofactor activity of thrombomodulin on membrane, phospholipids were extracted from the membrane fraction of cultured human endothelial cells and the composition was determined (Table 1). The proportions of PtdCho, PtdEtn and PtdSer in the total phospholipid were 50.5 ± 2.6 , 21.6 ± 4.8 , 11.4 ± 2.4 and $5.8 \pm 3.7\%$ respectively. The phospholipid ratios

Table 1 Phospholipid composition of membrane fraction in HUVECs

Results are expressed as percentage of total phospholipids in membrane fraction of HUVECs. Total phospholipid content was $360.1 \mu\text{g}$ of phospholipids/mg of protein. Data are means $\pm$ S.D. from three independent experiments.

Phospholipids	Percentage of total phospholipid
PtdCho	50.5 ± 2.6
PtdEtn	21.6 ± 4.8
PtdSer	11.4 ± 2.4
PtdIns	5.8 ± 3.7
Others	10.7 ± 4.5

Table 2 Effect of phospholipid vesicles extracted from HUVECs on thrombin-dependent Protein C activation by purified placental thrombomodulin

Phospholipids were extracted from cultured HUVECs and were separated into classes by t.l.c. as described in the Experimental section. Thrombomodulin was reconstituted into phospholipid vesicles containing 0.5 mg PtdCho/ml or a mixture of 0.25 mg PtdCho plus 0.25 mg of other phospholipids (PtdIns, PtdSer, PtdEtn)/ml and the effect of these phospholipid vesicles on the rate of activation of human Protein C by the thrombin–thrombomodulin complex was measured. The final reaction mixture contained 2 nM thrombomodulin, 20 nM thrombin, 2 mM CaCl_2 , 2 μM Protein C, 5 mg/ml BSA and various concentrations of phospholipids. Results are from two separate experiments and the numbers in parentheses are the relative values compared with no phospholipid.

Phospholipid vesicles	Protein C activation (pmol/min per nmol of thrombomodulin)	
No phospholipid	20.1 (1)	26.3 (1)
PtdCho	26.0 (1.3)	30.0 (1.1)
PtdIns/PtdCho	46.6 (2.3)	40.4 (1.5)
PtdSer/PtdCho	94.5 (4.7)	103.5 (3.9)
PtdEtn/PtdCho	171.5 (8.5)	218.2 (8.3)

were comparable with those of other eukaryotes, with some exceptions [35,36]. The specificity of phospholipid classes which could accelerate the cofactor activity of thrombomodulin for thrombin-dependent Protein C activation was investigated by measuring the amidolytic activity of activated Protein C (Table 2). Phospholipids extracted from HUVECs were used after separation into phospholipid classes. Phospholipid vesicles were prepared at a 1:1 (w/w) ratio of each phospholipid to PtdCho to avoid artificial effects of vesicle construction on thrombomodulin cofactor activity. Thrombomodulin reconstituted into phospholipid vesicles consisting of PtdCho alone or PtdIns plus PtdCho (PtdIns/PtdCho) increased the cofactor activity by 1.2- and 1.9-fold over the control respectively at a concentration of 0.5 mg of total phospholipid/ml of reaction mixture. On the other hand, 0.5 mg/ml of PtdSer/PtdCho and PtdEtn/PtdCho vesicles markedly increased the activity 4.3- and 8.4-fold respectively over that in the absence of phospholipid. These increases in activity were dependent on the concentration of PtdEtn or PtdSer up to 0.25 mg/ml (total 0.5 mg/ml). When rabbit lung thrombomodulin was used instead of that from human placenta, almost the same behaviour with respect to the phospholipid composition was observed (results not shown). The increase in activity was completely blocked when thrombomodulin was preincubated with anti-thrombomodulin IgG before reconstitution into the vesicles. These phospholipids did not directly affect either Protein C activation or the proteolytic activity of activated Protein C, as Protein C or activated Protein C mixed with the vesicles consisting of the respective phospholipid had the same activity as those without phospholipid (results not shown). These results indicate that PtdSer and PtdEtn greatly increase the cofactor activity of thrombomodulin for thrombin-dependent Protein C activation.

PtdEtn containing unsaturated fatty acid augments cofactor activity of thrombomodulin for thrombin-dependent Protein C activation

The effect of the fatty acid composition of the phospholipid on the cofactor activity of thrombomodulin was studied (Figure 1). Authentic phospholipids containing stearic acid, linoleic acid and linolenic acid at the C-1 and C-2 positions of the *sn*-glycerol moiety were used. When thrombomodulin was reconstituted into distearoyl (18:0–18:0) PtdCho vesicles, no significant change in cofactor activity was observed compared with the control, which contained no phospholipids in the reaction mixture. An increase in degree of unsaturation of the fatty acid in PtdCho produced a slight increase in cofactor activity, i.e. the activity of thrombomodulin reconstituted into dioleoyl (18:1–18:1) PtdCho/18:0–18:0 PtdCho, dilinoleoyl (18:2–18:2) PtdCho/18:0–18:0 PtdCho, dilinolenoyl (18:3–18:3) PtdCho/18:0–18:0 PtdCho vesicles increased 2.1-, 2.9- and 3.2-fold respectively with respect to that reconstituted into 18:0–18:0 PtdCho alone. Although vesicles consisting of 18:0–18:0 PtdEtn/18:0–18:0 PtdCho did not alter cofactor activity compared with that in the absence of phospholipid, the activity markedly increased with increasing degree of unsaturation of the fatty acid in PtdEtn. Vesicles consisting of 18:1–18:1 PtdEtn/18:0–18:0 PtdCho, 18:2–18:2 PtdEtn/18:0–18:0 PtdCho and 18:3–18:3 PtdEtn/18:0–18:0 PtdCho increased the activity approximately 9.4-, 11.7- and 14.6-fold over that in 18:0–18:0 PtdEtn/18:0–18:0 PtdCho, and the activity in unsaturated PtdEtn/18:0–18:0 PtdCho was four to five times that in the respective unsaturated PtdCho/18:0–18:0 PtdCho. In order to discover the amount of thrombomodulin antigen reconstituted into phospholipid vesicles of different composition, sucrose-density-gradient centrifugation was performed. No significant difference in the recovery of thrombo-

Figure 1 Effect of the degree of unsaturation of fatty acid in PtdEtn and PtdCho vesicles on thrombin-dependent Protein C activation by thrombomodulin

Thrombomodulin purified from human placenta was reconstituted into phospholipid vesicles consisting of authentic PtdEtn and PtdCho containing stearic acid, linoleic acid or linolenic acid at both the C-1 and C-2 positions of the *sn*-glycerol moiety. The assay conditions were as described in Table 2. Data are means $\pm$ S.D. from four independent experiments. $\odot$, 18:0–18:0 PtdCho alone or 18:0–18:0 PtdEtn/18:0–18:0 PtdCho (1:1, w/w); $\circ$, 18:1–18:1 PtdCho/18:0–18:0 PtdCho (1:1, w/w); $\triangle$, 18:2–18:2 PtdCho/18:0–18:0 PtdCho (1:1, w/w); $\square$, 18:3–18:3 PtdCho/18:0–18:0 PtdCho (1:1, w/w); $\bullet$, 18:1–18:1 PtdEtn/18:0–18:0 PtdCho (1:1, w/w); $\blacktriangle$, 18:2–18:2 PtdEtn/18:0–18:0 PtdCho (1:1, w/w); $\blacksquare$, 18:3–18:3 PtdEtn/18:0–18:0 PtdCho (1:1, w/w).

modulin was observed in the 0–15% sucrose fraction between phospholipid vesicles prepared with 18:0–18:0 PtdCho alone or those prepared with a mixture of 18:0–18:0 PtdCho and other phospholipids at a 1:1 (w/w) ratio. These results indicate that thrombomodulin reconstituted into PtdEtn containing unsaturated fatty acid has greater cofactor activity than that reconstituted into PtdCho containing unsaturated fatty acid. On the other hand, an unsaturated fatty acid, such as oleic acid itself, emulsified in 18:0–18:0 PtdCho vesicles had no effect on cofactor activity (results not shown).

Table 3 Kinetic parameters of thrombomodulin reconstituted into PtdEtn/PtdCho

The rate of activation of Protein C by the thrombin–thrombomodulin complex was measured under the condition of either change in the concentration of thrombin, Protein C or Ca^{2+} . A final volume of 100 μl of the reaction mixture contained 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 5 mg/ml BSA, 0.2–20 nM thrombin, 0.067–5 μM Protein C, 0.1–3 mM CaCl_2 and either 2 nM thrombomodulin without phospholipid or after reconstitution of thrombomodulin into phospholipid vesicles containing a mixture of 0.25 mg 18:3–18:3 PtdEtn/ml plus 0.25 mg 18:0–18:0 PtdCho/ml at a lipid/protein molar ratio of 316500:1 as described in the Experimental section. Concentrations of thrombin and Protein C leading to half-maximal rate of the activation were determined by double-reciprocal plot of the data in Figures 2(a) and 2(b), and those of Ca^{2+} were determined by the substrate concentration curve in Figure 3. The maximal rate of the activation was determined by the average of data in Figures 2(a) and 2(b).

	Thrombin K_d (nM)	Protein C K_m (μM)	Ca^{2+} K_s (mM)	Protein C activation $V_{\max}$ (pmol/min per nmol)
No phospholipid	1.82	1.84	0.25	17.8
PtdEtn/PtdCho	0.86	0.18	0.5–1.0	253.1

Figure 2 Thrombin and Protein C concentration-dependence of Protein C activation by the thrombin–thrombomodulin complex

Activation of Protein C was measured in a reaction mixture containing 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 5 mg/ml BSA, 2 mM CaCl_2 , 5 μM Protein C (a), 20 nM thrombin (b) and 2 nM thrombomodulin or that reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho (0.25 mg plus 0.25 mg/ml) vesicles. Thrombin concentration was varied between 0.2 and 20 nM (a) and Protein C concentration was varied between 0.067 and 5 μM (b). $\square$ and $\circ$, thrombomodulin; $\blacksquare$ and $\bullet$, thrombomodulin reconstituted into PtdEtn/PtdCho vesicles.

Figure 3 Ca^{2+} -dependence of Protein C and Gla-domainless Protein C activations by the thrombin–thrombomodulin complex

Activation of Protein C and Gla-domainless Protein C was measured in reaction mixture containing 2 nM thrombomodulin or that reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho (0.25 mg plus 0.25 mg/ml) vesicles, 5 mg/ml BSA, 20 nM thrombin, 2 μM Protein C or Gla-domainless Protein C and various concentrations of CaCl_2 . $\square$, Protein C activation with thrombomodulin; $\blacksquare$, Protein C activation with thrombomodulin reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho; $\circ$, Gla-domainless Protein C activation with thrombomodulin; $\bullet$, Gla-domainless Protein C activation with thrombomodulin reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho.

Ca^{2+} -dependence of Protein C activation and interactions between thrombin, thrombomodulin, Protein C and PtdEtn

Kinetic parameters in the assay system of thrombomodulin cofactor activity were measured for thrombomodulin reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho vesicles (Figure 2 and Table 3). The dissociation constant (K_d) for thrombin of thrombomodulin was reduced to half of that without phospholipid and the Michaelis constant (K_m) for Protein C of thrombomodulin was decreased to one-tenth compared with no phospholipid. On the other hand, the Ca^{2+} concentration required for the half-maximal rate of activation was increased. The Ca^{2+} -dependence of activation of Protein C or Gla-domainless Protein C was measured with soluble thrombomodulin or thrombo-

Figure 4 Protein C or Gla-domainless Protein C activation by thrombin-thrombomodulin or thrombin-transmembrane-domainless thrombomodulin complexes

(a) Native thrombomodulin or r-thrombomodulin was suspended in buffer A containing 18:3-18:3 PtdEtn/18:0-18:0 PtdCho (0.25 mg plus 0.25 mg/ml) vesicles for more than 30 min at 20-25 °C and Protein C activation was measured or (b) native thrombomodulin was suspended in buffer A containing the phospholipid vesicles and Protein C or Gla-domainless Protein C activation was measured. r-Thrombomodulin (491 amino acids) lacks both the transmembrane and cytoplasmic domains of endogenous thrombomodulin (557 amino acids). Gla-domainless Protein C was prepared by proteolytic removal of 41 amino acids from the N-terminus of the light chain of human Protein C (405 amino acids). The assay conditions were the same as those described in Table 2. (a) Protein C activation by native thrombomodulin (●) and transmembrane-domainless thrombomodulin (○); (b) activation of Protein C (●) and Gla-domainless Protein C (◻) by native thrombomodulin.

modulin reconstituted into 18:3-18:3 PtdEtn/18:0-18:0 PtdCho (Figure 3). The Ca^{2+} -dependence of the Protein-C activation system showed a hyperbolic curve in the absence of phospholipid, whereas it was sigmoidal in the presence of phospholipid vesicles and the activity was highest at the physiological concentration of serum Ca^{2+} (2-3 mM). The Ca^{2+} -dependence of Gla-domainless Protein C activation fitted a simple hyperbolic curve irrespective of the presence of vesicles. The results in Figures 2 and 3 and Table 3 indicate that PtdEtn containing unsaturated fatty acid accelerates interactions between thrombin, thrombomodulin and Protein C in the presence of the plasma level of Ca^{2+} .

In order to elucidate the mechanisms of increase in thrombin-dependent Protein C activation by thrombomodulin reconstituted into 18:3-18:3 PtdEtn/18:0-18:0 PtdCho, cofactor activity was investigated by using r-thrombomodulin lacking both transmembrane domain and cytosolic domain compared with native thrombomodulin or Gla-domainless Protein C (Figure 4). The increase in cofactor activity in 18:3-18:3 PtdEtn/18:0-18:0 PtdCho vesicles was completely abolished when r-thrombomodulin was used instead of native thrombomodulin (Figure 4a) or when Gla-domainless Protein C was used instead of native Protein C (Figure 4b). These results not only support the previous finding that interaction between the transmembrane domain of thrombomodulin and phospholipid vesicle is essential for the enhancement of cofactor activity [37], but also indicate that the Gla-domain of Protein C plays a key role in the PtdEtn-dependent augmentation of cofactor activity.

In order to examine the binding efficacies of native Protein C and Gla-domainless Protein C on PtdEtn/PtdCho, vesicles of phospholipid with different degrees of unsaturation of the fatty acid were fixed on a microwell plate and the amount of Protein C or Gla-domainless Protein C bound to each phospholipid was determined. In the absence of Ca^{2+} , native Protein C scarcely bound to PtdEtn regardless of the degree of unsaturation (Figure 5a). No significant difference in Protein C binding to 18:0-18:0

Figure 5 Binding of Protein C, Gla-domainless Protein C and thrombomodulin on PtdEtn/PtdCho vesicles in the presence or absence of Ca^{2+}

Phospholipid vesicles consisting of 18:0-18:0 PtdCho alone (○, □) or 18:0-18:0 PtdEtn/18:0-18:0 PtdCho (1:1, w/w, △, ▽), 18:1-18:1 PtdEtn/18:0-18:0 PtdCho (1:1, w/w, ◇, ▹), 18:2-18:2 PtdEtn/18:0-18:0 PtdCho (1:1, w/w, ●, ▢) and 18:3-18:3 PtdEtn/18:0-18:0 PtdCho (1:1, w/w, ○, □) were fixed on a microwell plate and the amounts of Protein C (a) and thrombomodulin (b) bound were measured. Phospholipid vesicles were coated on a microplate and washed with buffer B containing 1% (w/v) BSA as described in the Experimental section. Protein C (50-400 nM final concentration) or thrombomodulin (1-10 nM) was added to each well in the absence (open symbols) or presence (closed symbols) of 2 mM CaCl_2 . After washing of the plate, the amount of each protein bound to phospholipid vesicles was measured with monoclonal antibody to the respective protein as described in the Experimental section.

PtdEtn/18:0-18:0 PtdCho was observed compared with 18:0-18:0 PtdCho in the presence or absence of 2 mM CaCl_2 . In the presence of 2 mM Ca^{2+} , Protein C binding to each phospholipid vesicle increased with increasing concentration of Protein C. Proportional increases in binding were observed in the range 0-200 nM Protein C, and the slope, which reflects the ability of Protein C to bind to phospholipid, increased with an increase in the unsaturation of fatty acid in PtdEtn in the presence of 2 mM CaCl_2 (Figure 5a). In contrast, Gla-domainless Protein C did not bind to wells coated with any of the phospholipid vesicles and the binding was not increased even in the presence of Ca^{2+} (results not shown). Thrombomodulin binding to PtdEtn vesicles as a

Figure 6 Exposure of PtdEtn on the surface of human endothelial cells after stimulation with thrombin

Cultured HUVECs (60 mm dish) were treated with 0.5 NIH unit/ml thrombin (or buffer in control experiments) in the presence of 0.2 mM CaCl_2 for 20 min and were allowed to react further with 40 mM TNBS for 40 min. The reaction was terminated by the addition of acetic acid, and phospholipids were extracted with chloroform/methanol (2:1, v/v). PtdEtn and TNBS-reactable PtdEtn were separated by t.l.c. and phosphorus contents were measured. Results represent the means of four independent experiments and are expressed as percentage PtdEtn content with respect to total phospholipid. ■, PtdEtn allowed to react with TNBS; ▨, PtdEtn not allowed to react with TNBS.

function of thrombomodulin concentration was studied using the same procedure as for Protein C (Figure 5b). Thrombomodulin binding to each phospholipid vesicle increased with the increase in protein concentration, and proportional increases in binding were observed in the range 1–5 nM thrombomodulin. The affinity between thrombomodulin and phospholipid is also reflected by the slope. With the increase in the degree of unsaturation of the fatty acid in PtdEtn, the slope increased irrespective of the absence of 2 mM Ca^{2+} . The amount of thrombomodulin bound to phospholipid vesicles was also determined by measuring the cofactor activity of thrombomodulin directly, and the same result was obtained as when it was measured by using antibody (results not shown). When transmembrane-domainless r-thrombomodulin was used, no binding was observed even when 18:3–18:3 PtdEtn/18:0–18:0 PtdCho vesicles were fixed to the well (results not shown). Thus it became apparent that the PtdEtn-dependent increase in cofactor activity of thrombomodulin was correlated with binding of both Protein C to PtdEtn and thrombomodulin to PtdEtn, and it was also clear that the Gla domain of Protein C was essential for the Ca^{2+} -dependent interaction between Protein C and phospholipid.

Outside–Inside translocation of PtdEtn in endothelial cells

It has been shown that aminophospholipids are primarily localized on the plasma membrane interior in a variety of cells,

although some PtdEtn is found on the cell surface [38]. To evaluate the physiological significance of PtdEtn for Protein C activation on the surface of HUVECs, the cells were stimulated with thrombin and PtdEtn localization was investigated (Figure 6). After treatment of HUVECs for 20 min with thrombin (0.5 units/ml), TNBS, a membrane-impermeant agent, was added to the culture, and the amount of PtdEtn that could bind to TNBS was determined [26]. Without stimulation of HUVECs, approximately four-fifths of PtdEtn was localized on the inside of the membrane, as 4.3% and 17.3% of total phospholipids were PtdEtn reacted and not reacted with TNBS respectively. In the cells treated with thrombin, TNBS-reactable PtdEtn increased to 14.8% of total phospholipid, indicating that two-thirds of the PtdEtn was localized at the outer surface of the cells after thrombin stimulation. It was confirmed by means of the Trypan Blue exclusion test that, under the conditions employed, there was no difference in membrane integrity between thrombin-stimulated and control HUVECs. These results suggest that PtdEtn appeared on the outside of the plasma membrane of HUVECs when they were stimulated with physiological compounds such as thrombin.

DISCUSSION

Thrombomodulin is a glycoprotein on the surface of endothelial cells, which acts as a cofactor to accelerate the rate of thrombin-catalysed Protein C activation [4–7]. Studies on the environment of thrombomodulin on the surface of the plasma membrane are important in view of the physiological function of the molecule. In the present study, we investigated the effects of phospholipid classes and fatty acid composition on thrombin-dependent Protein C activation by thrombomodulin (cofactor activity of thrombomodulin). We found that thrombin–thrombomodulin-catalysed activation of Protein C was 8.4-fold higher in vesicles containing PtdEtn/PtdCho extracted from HUVECs than in the absence of phospholipid. The largest increase in cofactor activity of thrombomodulin by phospholipid (PtdSer/PtdCho) vesicles previously reported was 3-fold [13]. Therefore we focused on the effect of fatty acid composition of phospholipid on cofactor activity of thrombomodulin using PtdEtn.

The ratio of PtdEtn/PtdCho (10–50 μg of phospholipid/reaction mixture) to thrombomodulin (15 ng/reaction mixture) used in this series of experiments for measuring cofactor activity is comparable with that found in the HUVECs, as we [10] and Bartha et al. [39] have shown that total thrombomodulin antigen was approx. 150–200 ng per 10^6 HUVECs, which is approx. equivalent to 160 μg of total phospholipid (in crude membrane fraction) per 10^6 HUVECs in the present study. Under these conditions, the cofactor activity of thrombomodulin increased with an increase in degree of unsaturation of the fatty acid in the phospholipid, and a remarkable increase in activity was obtained when thrombomodulin was reconstituted into vesicles containing 18:3–18:3 PtdEtn. To examine the significance of the position of the unsaturated fatty acid in PtdEtn, thrombomodulin was reconstituted into 1-palmitoyl-2-oleoyl PtdEtn (16:0–18:1 PtdEtn)/18:0–18:0 PtdCho and its cofactor activity was compared with that in 18:1–18:1 PtdEtn/18:0–18:0 PtdCho vesicles. The cofactor activity in the two vesicles was the same (results not shown), suggesting that unsaturated fatty acid in the C-2 position of the *sn*-glycerol moiety might be important for cofactor activity.

The kinetic study indicated that thrombomodulin reconstituted into 18:3–18:3 PtdEtn/18:0–18:0 PtdCho vesicles showed a reduced dissociation constant for thrombin. As thrombin does not contain any domain which directly interacts with phospho-

lipids in the plasma membrane [40,41], the decrease in the dissociation constant might arise from a conformational change in thrombomodulin on interaction of its transmembrane domain with PtdEtn/PtdCho. In our experiment, PtdEtn/PtdCho did not directly affect the proteolytic activity of thrombin (results not shown). It has been shown that the insertion of a *cis* double bond at the 9,10 position of saturated fatty acid at the *sn*-2 position of pure phospholipids causes striking alterations, which arise because the double bond makes an angle of 7–8° to the normal bilayer [42,43]. As an ethanolamine residue is less bulky than a choline residue, a double bond in PtdEtn may allow a more flexible conformation of the hydrocarbon chains. Rothfield and Romeo [44] reported that the activity of certain membrane-bound enzymes depended critically on the presence of *cis* double bonds in phospholipid, indicating the existence of a specific interaction between enzymic recognition sites and the *cis* double bond. Therefore the activating role of the unsaturated fatty acid in phospholipid may depend on the unique configuration, and the cofactor activity of thrombomodulin may be controlled through modulation of the membrane fluidity.

The striking 18:3–18:3 PtdEtn-dependent increase in the affinity between thrombin–thrombomodulin complex and Protein C was reflected by a reduction in the K_m value for Protein C to one-tenth of that in the presence of 18:3–18:3 PtdEtn/PtdCho vesicles. In the absence of phospholipid, thrombin-dependent Protein C activation and Gla-domainless Protein C activation by thrombomodulin were essentially the same (Figure 3). This result suggests that Protein C bound to thrombomodulin at some region other than the Gla domain and was activated by the thrombin–thrombomodulin complex. In the presence of 18:3–18:3 PtdEtn/18:0–18:0 PtdCho vesicles, on the other hand, thrombin-dependent Protein C activation caused by thrombomodulin was markedly elevated in a sigmoidal manner with respect to Ca^{2+} concentration, whereas the Gla-domainless Protein C activation showed a typical saturation curve with respect to Ca^{2+} concentration, and the activation was the same as that in the absence of phospholipid. This result indicates that the increase in cofactor activity of thrombomodulin reconstituted into phospholipid vesicles is related to the presence of the Gla domain in Protein C and the elevation effectively occurs at the plasma concentration of Ca^{2+} . There are two inconsistent reports, one claiming that Protein C interacts directly with anionic phospholipids, leading to an increased affinity of Protein C for thrombin–thrombomodulin [19] and the other claiming that Protein C does not directly interact with phospholipids and binds to thrombomodulin that is conformationally changed by interaction with phospholipids [20,45]. Our binding study (Figure 5) shows that binding of Protein C to PtdEtn increased with an increase in degree of unsaturation of the fatty acid in the presence of Ca^{2+} , although Gla-domainless Protein C did not bind to PtdEtn in the presence or absence of Ca^{2+} . Therefore our results indicate that the Gla domain of Protein C interacts directly with PtdEtn in a Ca^{2+} -dependent manner and facilitates the rapid activation of Protein C on phospholipid vesicles. We cannot exclude the possibility that the Gla domain of Protein C also recognizes thrombomodulin in a Ca^{2+} -independent manner in addition to phospholipids on the membrane surface in a Ca^{2+} -dependent manner. Furthermore, the mechanism by which unsaturated fatty acid of PtdEtn increases the binding of Protein C to phospholipid vesicles is unknown.

It is generally accepted that mammalian plasma membranes display a stable asymmetrical distribution of lipids across the bilayer. Aminophospholipids PtdEtn and PtdSer are preferentially located at the cytoplasmic surface of various plasma membranes [38]. In spite of the fact that PtdEtn was mainly

localized in the inner membrane of HUVECs in our experiment, an increase in surface exposure of PtdEtn after thrombin treatment was observed. In platelets, transbilayer asymmetry is rapidly lost on activation by certain platelet agonists [46–49]. Bevers et al. [48] demonstrated that stimulation of platelets with the Ca^{2+} ionophore A23187 or the combined action of collagen plus thrombin resulted in a rapid outer-surface appearance of PtdSer, which was almost entirely located on the cytoplasmic side of the plasma membrane of non-activated human platelets. On the surface of endothelial cells, the rate of thrombin formation by the enzyme complex, Factor Xa–Factor Va, is also essentially dependent on the presence of an anionic phospholipid-containing surface [40]. In this respect, both pro- and anti-coagulation activities would be accelerated after exposure of PtdEtn and PtdSer at the surface of the plasma membrane. The present results indicate that partial loss of the asymmetric orientation of PtdEtn occurs in stimulated endothelial cells and this leads to the formation of an anticoagulant surface on the plasma membrane of endothelial cells, although the balance between pro- and anti-coagulation on the membrane surface must be essentially dependent on the expression of the factors relating to pro- and anti-coagulation [11]. On the basis of the topological data presented here and the fact that endothelial plasma membrane contains a large amount of unsaturated phospholipids (e.g. arachidonic acid is predominant at the C-2 position of PtdEtn) [21,50], it is reasonable to conclude that both the composition and distribution of phospholipids in the membrane modulate the pro- and anti-thrombotic state.

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