Endothelial Thrombomodulin Induces Ca$^{2+}$ Signals and Nitric Oxide Synthesis through Epidermal Growth Factor Receptor Kinase and Calmodulin Kinase II*  

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Endothelial membrane-bound thrombomodulin is a high affinity receptor for thrombin to inhibit coagulation. We previously demonstrated that the thrombin-thrombomodulin complex restrains cell proliferation mediated through protease-activated receptor (PAR)-1. We have now tested the hypothesis that thrombomodulin transduces a signal to activate the endothelial nitric-oxide synthase (NOS3) and to modulate G protein-coupled receptor signaling. Cultured human umbilical vein endothelial cells were stimulated with thrombin or a mutant of thrombin that binds to thrombomodulin and has no catalytic activity on PAR-1. Thrombin and its mutant dose dependently activated NO release at cell surface. Pretreatment with anti-thrombomodulin antibody suppressed NO response to the mutant and to low thrombin concentration and reduced by half response to high concentration. Thrombin receptor-activating peptide that only activates PAR-1 and high thrombin concentration induced marked biphasic Ca$^{2+}$ signals with rapid phosphorylation of PLC$_{\gamma1}$ and NOS3 at both serine 1177 and threonine 495. The mutant thrombin evoked a Ca$^{2+}$ spark and progressive phosphorylation of Src family kinases at tyrosine 416 and NOS3 only at threonine 495. It activated rapid phosphatidylinositol-3 kinase- and calmodulin kinase II. Complete epidermal growth factor receptor inhibition only partly reduced the activation of phospholipase C$_{\gamma1}$ and NOS3. Prestimulation of thrombomodulin did not affect NO release but reduced Ca$^{2+}$ responses to thrombin and histamine, suggesting cross-talks between thrombomodulin and G protein-coupled receptors. This is the first demonstration of an outside-in signal mediated by the cell surface thrombomodulin receptor to activate NOS3 through tyrosine kinase-dependent pathway. This signaling may contribute to thrombomodulin function in thrombosis, inflammation, and atherosclerosis.

Thrombomodulin (TM)$^4$ is a transmembrane glycoprotein synthesized by endothelial cells and a membrane receptor for thrombin. It consists of an amino-terminal domain homologous to C-type lectins, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr-rich sequence, a transmembrane domain, and a short cytoplasmic tail (1, 2). Thrombin binds to the EGF-like domains 5 and 6 of TM to form a high affinity complex (3). The latter is essential for hemostasis regulation by accelerating the activation of protein C, a physiological inhibitor of coagulation (2), and TAFI (thrombin-activable fibrinolysis inhibitor), an endogenous inhibitor of fibrinolysis (4).

Thrombin also plays a key role in vessel wound healing and recanalization. It induces multiple phenotypic changes of blood and vascular cells to affect vascular tone, cell permeability and growth, and leukocyte trafficking (5). Thrombin mediates cellular events by signal transduction through protease-activated receptors (PARs) (6). It activates PAR-1 by cleavage of its N terminus extracellularly, thereby unmasking the tethered ligand SELLNRN that flips over to activate the receptor. We have previously shown that the thrombin-TM complex restrains endothelial cell proliferation activated by the synthetic peptide SELLRN, so-called thrombin receptor-activating peptide (TRAP) (7). The binding of thrombin to endothelial TM indeed decreased the DNA synthesis by prolonging the phosphorylation and the nuclear retention of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (8).

Phosphorylation of ERK1/2 is dependent on nitric oxide (NO) generated by NO synthase (NOS) (9). Through ERK1/2 phosphorylation, NO mediates either proliferative or anti-proliferative responses (10–12). As a messenger, NO is also involved in vascular endothelial growth factor-mediated angiogenesis (13, 14). It was recently reported that a human recombinant TM containing the six EGF-like domains and the Ser/Thr-rich sequence induces angiogenesis through activation of the endothelial NOS (NOS3) and ERK1/2, as do growth factors (15). We previously demonstrated that thrombin activates NOS3 in human umbilical vein endothelial cells (HUVECs) (16). The purpose of the present study was to investigate whether thrombin binding to endothelial membrane-bound TM induced signaling events to activate NOS3 and modulate G protein-coupled receptor (GPCR) signals. Our tool was a mutant of thrombin that binds to TM but has no catalytic activity on PAR-1. We report that endothelial TM mediates Ca$^{2+}$ spark and NO synthesis through the EGF receptor (EGFR) kinase and calmodulin kinase II (CaMKII).

MATERIALS AND METHODS  

Reagents and Antibodies—Primers, TRIZol, and culture medium were purchased from Invitrogen. Fura-2 acetoxymethyl ester (fura-2 AM) and Alexa 488-conjugated anti-mouse IgG antibody were obtained from Molecular Probes (Eugene, OR). TRAP was purchased from Bachem. The thrombin mutant S195A, which has no catalytic activity but binds to TM, was provided by Dr. B. Le Bonniec (INSERM Unit 428, Paris, France) (17). Human thrombin and the selective inhibitors of phosphatidylinositol-3 kinase (PI3K) (LY294002), EGFR kinase...
(AG1478), Src family kinases (PP2), protein kinase C (PKC, Go6976 and PKC, rottlerin) and CaMKII (KN62) were from Calbiochem. The mouse monoclonal antibody against the EGF5–6 region of TM was from American Diagnostica Inc. (Greenwich, CT). The rabbit polyclonal antibodies against phosphor-Thr195, eNOS, phospho-Tyr1176, EGFR, and PLCγ1, the mouse monoclonal antibody against Src and the anti-rabbit IgG antibody linked to horseradish peroxidase were from Cell Signaling Inc. (Beverly, MA). The rabbit polyclonal antibodies against phospho-Thr286, CaMKII, and eNOS were from BD Biosciences. The rabbit polyclonal antibodies against phospho-Thr195, CaMKIIa and CaMKII and horseradish peroxidase-linked anti-mouse IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture—Endothelial cells were isolated from segments of human umbilical cord vein and cultured as previously described (18). At confluence, HUVECs were detached with trypsin/versene (in vitro), washed, and grown for 3–4 days. At subconfluence, cells were serum starved (0.5% (v/v) fetal calf serum) for 18 h. Cells were incubated in phosphate buffer, pH 7.4, containing 5 mM glucose, 0.5 mM MgCl2, and 1 mM CaCl2 (PBS-MgCa), except for Ca2+ measurements for which a specific reaction buffer was used (in mM: NaCl 136, KCl 5, Na2PO4 2, MgSO4 0.4, NaHCO3 4, CaCl2 1, glucose 8, glutamine 2, a mixture of amino acids, and HEPES 25, pH 7.4).

RT-PCR—Total RNA was extracted from HUVECs grown on 60-mm plastic dishes using the TRizol® reagent according to the manufacturer’s instructions. The cDNA was synthesized from 1 μg of total RNA by incubation for 15 min at 42 °C with 2.5 units/ml murine leukemia virus reverse transcriptase (PE Applied Biosystems) in 20 μl of PCR buffer II containing 5 mM MgCl2, 1 mM of deoxy-NTP, 1 unit/μl ribonuclease inhibitor, and 2.5 mM random hexamers. PCR was performed by using 3 l, and each primer at 0.2 l, and the following primers: eNOS sense primer, 5′-GAGGAAGGAGTCCAGTAACACAGC-3′; eNOS antisense primer, 5′-GGACTTGTGCTGTTGAGTTTCC-T′ (438-bp product). The PCR reaction mixture (25 μl) contained 2 μM MgCl2, PCR buffer II, AmpliTaq DNA polymerase (PE Applied Biosystems) at 25 milliunits/μl, and each primer at 0.2 μM. Amplification was performed in a programmable thermal controller (model PTC-100; MJ Research Inc.). Sample denaturation at 95 °C for 2 min was followed by 35 PCR cycles of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 72 °C, and a further incubation of 7 min at 72 °C after the last cycle. Each sample (5 μl) was electrophoresed on polyacrylamide gels (4–20% Tris/boric acid/EDTA) and stained for 15 min with ethidium bromide (2.5 μg/ml) for densitometric analysis.

Western Blot—Proteins of cell homogenates were resolved by SDS-PAGE as previously described (19). The proteins transferred onto nitrocellulose membranes were incubated overnight at 4 °C with primary mouse- or rabbit-specific antibody (1:1000 dilution), rinsed, and then incubated at room temperature for 90 min with 1:2000 dilution of horseradish peroxidase-linked anti-mouse or anti-rabbit IgG secondary antibody, respectively. Membranes were reprobed with antibodies against unphosphorylated proteins.

Measurements of Nitric Oxide—The NO released at the surface of cells grown on 35-mm plastic culture dishes was measured by differential pulse amperometry at a porphyrinic NO-selective microsensor as previously described (20). The NO sensor was calibrated by the addition of NO standard solutions. Treatment of HUVECs with kinase inhibitors did not change the NO calibration curves. Results were expressed as the maximum of the agonist-induced oxidation current.

Results

The amplitude of NO release at cell surface rose with increasing concentrations of both thrombin and the mutant thrombin S195A (Fig. 1, A and B). Thrombin activated NO synthesis with an EC50 of 0.2 (0.1–0.5) nM and S195A with an EC50 of 20 (7–70) nM. Noteworthy, the dose-response curve for thrombin was biphasic with a first plateau between 0.5 and 2 nM and a second one at 20 nM, suggesting that the two receptors TM and PAR-1 are involved in NOS3 activation (Fig. 1A). In contrast, the dose-response curve for the mutant thrombin was characterized by only one plateau of saturation between 200 nM and 1 μM (Fig. 1B). Cell pretreatment with an anti-TM antibody totally suppressed the NO release induced by 200 nM S195A and 0.5 nM thrombin but decreased by only 50% that activated by saturating concentration of thrombin (Fig. 1C). The results demonstrate the stimulation of NO synthesis by thrombin binding to TM at concentrations below the nanomolar range and to PAR-1 at upper concentrations.

To investigate the transduction pathway of TM and its role in the regulation of PAR-1 signaling, we used the maximal concentrations of 200 nM for S195A and 20 nM for thrombin. Because agonist-activated NO synthesis is dependent on Ca2+ (18), we first examined intracellular
Ca\(^{2+}\) signaling. Both NO synthesis and Ca\(^{2+}\) signals activated by the mutant thrombin S195A, thrombin, or the PAR-1-activating peptide TRAP were maximal at 15–30 s and rapidly decreased thereafter (Fig. 2). The maximum NO production was 21 \pm 2 \text{nM} after stimulation by S195A, 19 \pm 2 \text{nM} after thrombin, and 17 \pm 2 \text{nM} after 100 \text{\mu M} TRAP, a concentration that induced maximal Ca\(^{2+}\) signals in HUVECs (22). The Ca\(^{2+}\) signals evoked by the three agonists were, however, of different amplitudes (Fig. 2B). After addition of S195A the peak value (57 \pm 14 \text{nM}) was followed by a decrease back to initial value. After thrombin and TRAP, the peak was of much higher amplitude (254 \pm 46 and 281 \pm 49 \text{nM}, respectively) and followed by a plateau well above initial value (50 \pm 11 and 61 \pm 8 \text{nM}, respectively). Cell treatment with the anti-TM antibody had no effect on the Ca\(^{2+}\) response to 20 \text{nM} thrombin (250 \pm 60 and 38 \pm 14 \text{nM for peak and plateau, respectively}). This indicates that thrombin-induced Ca\(^{2+}\) signal is mainly caused by PAR-1 stimulation. Because PAR-1-mediated Ca\(^{2+}\) signal results from PLC\(_{\beta}\) activation (6), we examined the phosphorylation of an upstream (PLC\(_{\beta}\)) and a downstream (NOS3) Ca\(^{2+}\) signal-related enzyme. Thrombin induced a rapid and sustained phosphorylation of Ser\(^{537}\)-PLC\(_{\beta3}\) (Fig. 3A). In contrast, the mutant thrombin S195A did not activate the PLC\(_{\beta3}\) As shown by the dose-response curve for thrombin (Fig. 3B), Ser\(^{537}\)-PLC\(_{\beta3}\) phosphorylation was detectable with 2 \text{nM} but was significant only at 20 \text{nM}. This suggests that another PLC isoform is responsible for Ca\(^{2+}\)-dependent activation of NOS3 by S195A and low thrombin concentrations. It has been reported that high thrombin concentration activates Ca\(^{2+}\)-dependent phosphorylation of Ser\(^{1177}\)-NOS3 (23). In the present study, 20 \text{nM} thrombin induced a transient Ser\(^{1177}\)-NOS3 phosphorylation with a maximum at 1 min followed by a return toward basal levels within 20 min (Fig. 4). The time courses obtained here for Ser\(^{1177}\) phosphorylation and also for NO release (Fig. 2A), i.e. in serum-starved HUVECs, were similar to those we previously observed in cells cultured with 20% serum (16). During the first 5 min, the Ser\(^{1177}\)-NOS3 phosphorylation activated by TRAP was similar to that induced by thrombin (Fig. 4). At 20 min, however, Ser\(^{1177}\)-NOS3 remained phosphorylated in TRAP-treated cells. No phosphorylation of Ser\(^{1177}\)-NOS3 occurred following S195A stimulation. The results suggest that the amplitude of TM-induced Ca\(^{2+}\) spark was too low to activate the kinase responsible for phosphorylation of Ser\(^{1177}\) and demonstrate that this residue is not involved in TM-mediated NOS3 activation.

In contrast to that of Ser\(^{1177}\), NOS3 phosphorylation at the Thr\(^{495}\) residue depended on serum concentration in culture medium (Fig. 5, A and B). Reducing the serum level from 20 to 0.5% decreased the phosphorylation ratio of unstimulated cells from 0.74 \pm 0.02 (Fig. 5A) to 0.03 \pm 0.01 (p < 0.001) (Fig. 5B). In the presence of serum, thrombin-induced Thr\(^{495}\)-NOS3 phosphorylation at 2 min (Fig. 5A), whereas in serum-starved cells, Thr\(^{495}\)-NOS3 phosphorylation started at 1 min (Fig. 5). Under the latter conditions, all three agonists, i.e. thrombin, the mutant thrombin S195A, and TRAP, progressively increased Thr\(^{495}\)-NOS3 phosphorylation up to a plateau reached at 5 min (Fig. 5C). From 1 to 20 min, the intensities of phosphorylation were, however, lower in S195A-stimulated HUVECs than in TRAP-activated cells (p < 0.001) and intermediate in thrombin-stimulated ones (Fig. 5C). The results show that factors present in serum regulate Thr\(^{495}\)-NOS3 phosphorylation and that TM mediates NOS3 phosphorylation at this residue.

Both PKC and PI3K are involved in NOS3 activation by growth factors (24). To analyze the role of these two kinases in NOS3 activation mediated by TM, we studied the effects of the PKC\(_{\delta}\) and PI3K inhibi-
tors, rottlerin and LY 294002, respectively. The two inhibitors reduced TRAP-activated NO release, but they had opposite effects in thrombin- and S195A-stimulated cells (Fig. 6). Thrombin-activated NO release was not affected by LY 294002 but was markedly reduced by rottlerin. In contrast, S195A-induced NO synthesis was not altered by rottlerin but was abolished by LY 294002, indicating that TM-mediated NOS3 activation depended on PI3K. The activities of PI3K and Src family kinases are tightly linked in endothelial cells (25). We thus analyzed the participation of Src kinases in TM-induced NO synthesis. Although the Src inhibitor PP2 (100 nM) had no effect on S195A-activated NO release detected at 20–30 s (20 ± 4 versus 18 ± 3 nM in untreated cells, n = 5), Src was progressively phosphorylated at Tyr416 by S195A (Fig. 7). Phosphorylation of Tyr416-Src was maximal at 5 min and remained stable over 20 min. Following thrombin, a phosphorylation peak appeared at 1 min and was followed by a decrease back to a plateau of similar intensity as that evoked by S195A. As Src family kinases are upstream activators of ERK1/2 (26, 27), we examined whether S195A activated this cascade. Thrombin rapidly increased ERK1/2 phosphorylation (281 ± 82% at 5 min, n = 4, p < 0.05), whereas S195A did not (19 ± 28%, n = 5). The results demonstrate that TM signaling involves PI3K-dependent NOS3 activation and Src phosphorylation.

Because thrombin binds to the EGF-like domains of TM (2) and Src participates in receptor tyrosine kinase signaling (26), we investigated whether the EGFR kinase and its downstream effectors PLCγ and
CaMKII contributed to the TM transduction pathway. As shown in Fig. 8A, significant phosphorylation of Tyr1068-EGFR, Tyr783-PLCγ1, and Thr286-CaMKIIα occurred in HUVECs stimulated for 30 s by the mutant thrombin S195A. The EGFR kinase inhibitor AG1478 reduced EGFR and PLCγ1 phosphorylation but had no effect on Thr286-CaMKIIα autophosphorylation (Fig. 8A). Interestingly, the S195A-activated NO release was inhibited by AG1478 and by KN62, a CaMKII inhibitor (Fig. 8B). The results demonstrate that TM induces NOS3 activation through the EGFR kinase and the CaMKII.

As shown above, thrombin activates NO synthesis through both TM and PAR-1. We previously demonstrated that TM negatively modulates PAR-1-induced activation of ERK1/2 cascade (8). In addition, the GPCR activators thrombin and histamine inhibit the EGF-mediated activation of PI3K/Akt cascade (28). To examine whether a cross-talk between TM and GPCR exists, we measured NO and Ca2+ responses to thrombin or to histamine after a previous stimulation with the mutant thrombin S195A, thrombin, or TRAP. When cells were prestimulated by S195A, the NO response to thrombin or histamine was not altered (Fig. 9A), indicating that TM signaling does not modulate NOS3 activation by GPCR activators. When cells were prestimulated by thrombin or by the PAR-1 agonist TRAP, thrombin-activated NO synthesis was reduced (Fig. 9A, left panel) but that induced by histamine was not (right panel), indicating that PAR-1 signal down-regulates TM-mediated NOS3 activation.

Concerning Ca2+ signals, the thrombin-induced Ca2+ peak was reduced following prestimulation by thrombin but not following pre-stimulation by S195A or TRAP (Fig. 9B, left panel). Interestingly, both Ca2+ peak and plateau evoked by histamine were decreased irrespective of the agonist used to engage TM, i.e. thrombin or S195A (right panel). The results demonstrate that TM mediates a signal able to modulate GPCR-induced Ca2+ signal.

Angiogenic factors such as vascular endothelial or platelet-derived growth factors, but not EGF, have been shown to increase NOS3 expression (29, 30). We thus examined whether TM activated NOS3 transcription. As demonstrated by RT-PCR, the expression of NOS3 mRNA remained unchanged by treatment for 4–18 h with thrombin or S195A (not shown). Similarly, the protein levels did not significantly vary in HUVECs treated for 18 h with thrombin or S195A (116 ± 19 or 124 ± 22% of control, respectively), suggesting that TM-mediated EGF signal does not modulate NOS3 expression.

**DISCUSSION**

We previously demonstrated that the high affinity thrombin-thrombomodulin complex restrains PAR-1-induced activation of ERK1/2 cascade (8). We have now shown that thrombin binding to the membrane-bound TM activated NO synthesis through the EGFR kinase and the CaMKII in human endothelial cells. Our results demonstrate for the
first time that the endothelial TM mediates an outside-in signal to activate NOS3 and modulate GPCR-induced Ca$^{2+}$ signaling.

Through its exosite I, thrombin binds to EGF-like domains 5 and 6 of its high affinity receptor TM (3) and to its low affinity receptor PAR-1 to activate its proteolysis (6). We observed a biphasic dose-response curve of NO release with detectable NO synthesis from 0.1 nM thrombin. The high affinity receptor responsible for NOS3 activation was saturated with 0.5 nM thrombin, totally inactivated by the anti-TM antibody, and independent of PLC$_{b1}$. Phosphorylation of PLC$_{b1}$ was detectable from 2 nM thrombin. The low affinity receptor involved in NOS3 activation was saturated with 20 nM thrombin and insensitive to anti-TM antibody. Its signaling is associated with PLC$_{b1}$ activation and a Ca$^{2+}$ signal similar to that induced by PAR-1 agonist peptide. In HUVECs, increases in phosphoinositide hydrolysis have been readily detected at 0.1 nM thrombin that induced by PAR-1 agonist peptide. In HUVECs, increases in phosphorylation of PLC$_{b1}$, CaMKII, and activate CaMKII through IP3-dependent Ca$^{2+}$ spark and formation of Ca-caldomin complex (Fig. 10, Ca-CaM). The NOS3 is in turn activated by Ca-CaM binding and by PI3K- and CaMKII-dependent phosphorylation. However, we observed that the inhibition of EGFR kinase did not prevent the Tyr$^{286}$ autophosphorylation of CaMKII. The Ca-CaM binding alone allows maximal activity and access of CaMKII to protein substrates, including NOS3, whereas Tyr$^{286}$ autophosphorylation converts the enzyme into a Ca-CaM-independent state (38). Here, complete EGFR inhibition did not abolish CaMKII autophosphorylation or the activation of PLC$_{b1}$, and NOS3. This indicates that the TM transduction pathway only partly depends on EGFR.

The soluble tyrosine kinases of Src family are involved in the signal transduction of EGFR (26, 37). Activation of TM by the mutant thrombin induced sustained Src phosphorylation. However, NO synthesis remained unchanged in the presence of the potent Src inhibitor even at high concentration, suggesting that NO is upstream from Src. Such a proposal is supported by the comparison of NO release and Tyr$^{286}$-Src phosphorylation kinetics. The TM-induced NO release was rapid and reached a maximum at 20–30 s, whereas Src phosphorylation was maximal between 5 and 20 min. This agrees with a NO-dependent and/or phosphorylations on serines 114, 615, 633, and 1177 and threonine 417 (human amino acids) (16, 19, 40–42). To stimulate its catalytic activity, GPCR agonists and growth factors evoke NOS3 phosphorylation at Ser$^{615}$, Ser$^{633}$, and Ser$^{1177}$ (16, 19, 41–43). The GPCR agonists mediate high Ca$^{2+}$ signal and rapid transient phosphorylation at Ser$^{615}$ and Ser$^{1177}$ (41, 42). In contrast, growth factors induced low Ca$^{2+}$ signal and progressive long lasting phosphorylation at Ser$^{615}$ and Ser$^{1177}$ (41, 44). In the present
study, the TM-induced NO synthesis occurred independently of NOS3 phosphorylation at Ser1177. Binding of mutant thrombin to TM activated Thr495 phosphorylation with, however, a kinetic different from that of NO release. Noteworthy, we observed that factors present in serum influence the Thr495 dephosphorylation/phosphorylation, suggesting participation of this residue in NO-regulated proliferative mechanisms. Long lasting incubation in a low serum medium (0.5%) resulted in dephosphorylation of Thr495 in unstimulated cells. Such an attenuated Thr495 phosphorylation was reported following PKC depletition in unstimulated cells (42). Growth factors present in serum may stimulate Thr495 phosphorylation and basal NOS3 activity in a PKC-dependent manner. In our study, neither the PKCα inhibitor rottlerin nor the PKCβ inhibitor Go6976 (not shown) had any effect on TM-induced NO release. Because PKC activates Thr495 phosphorylation (43), it is not unlikely that another residue than Thr495 may be responsible for rapid TM-dependent NOS3 activation.

Membrane-bound TM negatively modulates cellular functions associated with thrombin-induced PAR-1 activation, such as cell proliferation (7), and contractility (45). These regulatory processes may rely on cross-talks between TM and GPCRs. Both Ca2+ and NO are cellular messengers involved in signal transduction (46, 47). Using a monoclonal antibody against the thrombin-binding domain of TM (EGF5–6 region), we demonstrated that thrombin generates Ca2+ signal through PAR-1 and NO synthesis through both TM and PAR-1. A first TM stimulation reduced the Ca2+ signal subsequently activated by GPCR agonists (thrombin, histamine), not the NO release. Calcium is one of the key regulators of cell proliferation, especially through interactions with cascade of mitogen-activated protein kinases including ERK1/2 (46). Effectors of the TM signaling pathway may inhibit PAR-1-dependent Ca2+ signal and contribute to restraining thrombin-induced cell proliferation and vasoconstriction. The EGFR-dependent TM signaling may interfere with PAR-1 transduction pathway by a cross-talk, in the same way as tyrosine kinase receptors and G protein-coupled receptors are linked by transactivation mechanisms (48). That previous TM stimulation did not prevent GPCR agonists from activating NOS3 suggests the involvement of different kinases and phosphorylation sites. On one hand, TM activated PI3K- and CaMKII-dependent NO synthesis independently of Ser1177-NOS3 phosphorylation. On the other hand, thrombin and histamine activate NOS3 and Ser1177 phosphorylation independently of PI3K and CaMKII (23). Furthermore, a first PAR-1 stimulation inhibited subsequent thrombin activation of NO synthesis, not of Ca2+ signal. Altogether, the results confirm the existence of cross-talks between GPCR and TM and show the absence of PAR-1 desensitization. Because thrombin inhibits the EGFR-induced phosphorylation of the main downstream PI3K effector, Akt (28), transactivation of receptor tyrosine kinases through PAR-1 may prevent a subsequent activation of TM by thrombin.

The identification of the signaling pathway activated by the cell surface receptor TM is essential for understanding the physiological role of its soluble form. Plasma TM levels are inversely correlated with the development of new-onset coronary heart disease, suggesting that soluble TM may be cardioprotective (49, 50). Thrombin binding to endothelial TM could trigger outside-in signal and conversion of membrane TM into a soluble ligand. It has recently been demonstrated that the intramembrane protease rhomboids involved in EGFR signaling cleave TM at the top of its transmembrane domain (51). The regulatory function of the membrane-bound TM could be comparable with that of synecans, a family of heparan- and chondroitin-sulfate-carrying transmembrane proteins. These glycoproteins modulate cell-extracellular matrix interactions, growth factor signaling, and cell adhesion by mediating outside-in signal and shedding of their extracellular domain (52).

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REFERENCES
1. Sadler, J. E. (1997) Thromb. Haemostasis 78, 392–395
2. Esmen, C. T. (2003) Chest 124, 265S–325S
3. Ye, J., Liu, L. W., Esmen, C. T., and Johnson, A. E. (1992) J. Biol. Chem. 267, 11023–11028
4. Nesheim, M. (2003) Chest 124, (suppl.) 33S–39S
5. Minami, T., Sugiyama, A., Wu, S. Q., Abid, R., Kodama, T., and Aird, W. C. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 41–53
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