Thrombomodulin Prolongs Thrombin-Induced Extracellular Signal–Regulated Kinase Phosphorylation and Nuclear Retention in Endothelial Cells

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Abstract—On endothelial cells, thrombin binds to thrombomodulin (TM), an integral membrane-bound glycoprotein, and to protease-activated receptors (PARs). Thrombin binding to TM modulates endothelial cell and smooth muscle cell proliferation mediated through PAR1. We studied the phosphorylation and nuclear translocation of extracellular signal–regulated kinases (ERKs) 1 and 2 in human umbilical vein endothelial cells activated by thrombin. Thrombin and thrombin receptor–activating peptide (TRAP)-induced DNA synthesis were significantly inhibited by PD98059, an inhibitor of ERK phosphorylation. Immunoblots of phosphorylated ERKs (pERKs) and immunocytochemical studies of pERK localization revealed differences in the signal generated by thrombin and TRAP. After a short activation (15 minutes), the phosphorylation and the intracellular localization of pERKs were the same with the 2 agonists. After 4 hours, however, pERKs were visualized in the nuclei of thrombin-activated cells but barely detectable in TRAP-activated cells. Moreover, after 4 hours, the pERKs were visualized in the nuclei of cells stimulated by TRAP in the presence of a thrombin mutant that bound to TM, whereas they were around the nuclei in cells stimulated by thrombin in the presence of a monoclonal antibody preventing thrombin binding to TM. The results demonstrate that ERKs are involved in human umbilical vein endothelial cell DNA synthesis mediated by PAR agonists, that the duration of pERK nuclear retention is in inverse ratio to the mitogenic response, and that in addition to its role in the regulation of blood coagulation, TM acts as a thrombin receptor that modulates the duration of pERK nuclear retention and cell proliferation in response to thrombin. (Circ Res. 2001;88:681-687.)

Key Words: thrombin ▪ thrombomodulin ▪ human endothelial cells ▪ mitogen-activated protein kinase

Thrombin is a multifunctional serine protease generated at sites of vascular injury. Thrombin plays a key role in blood coagulation and thrombotic disorders. It acts as the central enzyme of the coagulation cascade by cleaving fibrinogen into fibrin and favoring its own production by activating several coagulation factors by limited proteolysis. Thrombin also regulates its own formation after binding to thrombomodulin (TM), an integral membrane-bound glycoprotein expressed on endothelial cells. TM acts as a cofactor of thrombin to activate protein C, a serine protease ensuring proteolytic inactivation of 2 coagulation factors, factor Va and factor VIIIa. Thrombin also interacts with a variety of cells mediating inflammatory and proliferative responses to vascular injury. For all protein and cellular interactions, thrombin has a recognition site and a catalytic active site. By the former, called the anion-binding exosite (ABE1), thrombin binds to specific negatively charged sequences. By the catalytic site, thrombin exerts its proteolytic activity.

On vascular endothelial cells, thrombin ABE1 binds to TM. This binding occurs through the epithelial growth factor (EGF)-like domains 4 and 5 of TM. Thrombin ABE1 also binds to the typical heptahelical thrombin receptor, the first member of protease-activated G protein–coupled receptor family (PAR1). Thrombin catalytic site cleaves the aminoterminal tail of PAR1 that flips over to effect receptor activation. Endothelial cell responses to thrombin include prostacyclin, von Willebrand factor secretion, and cell proliferation. Synthetic peptides that mimic this new aminoterminal, so-called thrombin receptor–activating peptides (TRAPs), are full agonists for receptor activation, even after thrombin cleavage.

Comparison of thrombin and TRAP as to their ability to induce proliferation has led to the postulation that TM modulates PAR1 activation. Overexpression of TM on smooth muscle cells downregulates proliferation (estimated by 3H thymidine incorporation into the DNA) induced by both TRAP and thrombin, and the degree by which cell proliferation was blocked is directly correlated with the level of TM expressed on the cell surface. On endothelial cells, the inhibition of thrombin binding to TM by a monoclonal
antibody 3E2, directed against the fourth EGF-like domain, resulted in an increase of thrombin mitogenic effect. In a wide range of tumoral cells, TM seems to exert an inhibitory effect on cell proliferation independently of its thrombin cofactor activity. Thus, TM not only acts as a cofactor for protein C activation to inhibit thrombin generation but also seems to modulate the mitogenic signal triggered by PAR1 activation.

The mitogenic signal induced by many stimuli occurs through mitogen-activated protein kinases (MAPKs), among which are the two extracellular signal–regulated kinases (ERKs) p42<sup>ERK</sup> and p44<sup>ERK</sup>. ERK1 and ERK2 are ubiquitous cytosolic serine-threonine kinases involved in cellular growth and proliferation. Their activation requires phosphorylation of their tyrosine and threonine residues. Phosphorylation of ERKs promotes their dimerization and nuclear translocation. Once in the nucleus, phosphorylated ERKs (pERKs) activate various transcription factors. Duration of ERK phosphorylation and nuclear translocation are thus determinant for cell responses. In fibroblasts, thrombin and TRAP activate ERKs to induce their proliferation. In endothelial cells, pERKs activate cytosolic phospholipase A<sub>2</sub> implicated in prostacyclin secretion.

With this as background, we studied the TM-modulating role on thrombin-induced nuclear translocation of pERKs. For this purpose, we used an antibody anti-TM to prevent thrombin binding to TM and a thrombin mutant with no catalytic activity but the ability to bind TM. Our study indicates that in human umbilical vein endothelial cells (HUVECs), PAR stimulation leads to ERK phosphorylation and nuclear translocation and TM downregulates PAR signal to enhance pERK nuclear retention.

Materials and Methods

**Materials**

Human purified thrombin was purchased from Diagnostica Stago. The synthetic TRAP (SFLLRN) and the anti-TM antibody 3E2 were from Serbio. S195A was provided by Dr B. Le Bonniec (Faculté de Pharmacie, Paris, France). Polyclonal anti-ERK antibodies were purchased from New England Biolabs. PD98059 was from Calbiochem. Peroxidase coupled and biotinylated anti-rabbit antibodies and [<sup>3</sup>H]-thymidine were purchased from Amersham. The amount of p44<sup>ERK1</sup> and p42<sup>ERK2</sup> proteins within each sample was controlled after stripping of the blot that was reprobed with a polyclonal anti-ERK1 and anti-ERK2 antibody (1:1000 in TBS) to ensure that equivalent amounts of ERKs were present.

**Immunocytological Analysis**

The localization of pERKs was studied as previously described for fibroblasts. HUVECs were seeded on gelatin-coated glass coverslips and activated as above. HUVEC activation was stopped by removal of the medium and washed in TBS (Tris 0.2 mol/L, NaCl 0.8 mol/L, pH 7.6). The cells were fixed in 50% vol/vol methanol and 50% vol/vol acetone at 20°C for 8 minutes. After the washes in TBS, cells were incubated overnight at 4°C with the anti–phospho-ERK antibody (1:200 in TBS containing 3% vol/vol BSA). The incubation was stopped by 6 washes in TBS, and cells were incubated with biotinylated anti-rabbit IgG for 1 hour. After 6 washes in TBS, cells were incubated with Texas Red streptavidin for 1 hour. After the last washing step, cells were fixed for 30 seconds in absolute ethanol, washed in TBS and covered with mounting medium with DIAP, and examined with excitation-emission filters for rhodamine and for DIAP.

**Statistical Analysis**

Results of mitogenic assay were compared using Student’s t test. For immunocytological analysis, at least 80 to 150 cells from at least 3 separate experiments were examined to quantify the amount of pERK visualized. Statistical analysis was performed using Student’s unpaired t test.

**Results**

**Effect of MEK Inhibitor PD98059 on <sup>3</sup>H-Thymidine Incorporation Into DNA Induced by TRAP or Thrombin**

To establish whether ERKs are involved in HUVEC proliferation induced by thrombin or TRAP, we studied the effect of the MAPK kinase (MEK) inhibitor PD98059. Cell proliferation was estimated by [<sup>3</sup>H]-thymidine incorporation. As previously shown, HUVEC proliferation is more important in response to TRAP than to thrombin, at optimal concentrations, ie, 10 nmol/L and 100 μmol/L for thrombin and TRAP, respectively (Figure 1). Increasing concentrations of PD98059 resulted in significant decrease in DNA synthesis induced by thrombin or TRAP. The inhibitory effect of 10 μmol/L PD98059 was >80% both with thrombin and TRAP. This concentration was ascertained to inhibit thrombin- and TRAP-induced ERK phosphorylation but not global tyrosin phosphorylation in HUVECs (data not shown). The results show that DNA synthesis induced by thrombin and TRAP involves the phosphorylation of ERKs in HUVECs.

**Kinetics of ERK Phosphorylation Induced by Thrombin and TRAP**

The phosphorylation of ERKs was analyzed by Western blotting using an antibody specific for the phosphorylated...
forms of p44\textsubscript{ERK1} and p42\textsubscript{ERK2}. After the addition of TRAP or thrombin, the two proteins were rapidly and heavily phosphorylated after 15 minutes (Figure 2, top). The maximum phosphorylation level was identical with the two agonists and was reached between 60 and 90 minutes (top left). After 3 hours of activation, ERKs were slightly less phosphorylated with TRAP than with thrombin, a difference even more pronounced after 4 and 5 hours (top right). The dephosphorylation of ERKs back to the basal level was complete after 5 hours with TRAP and 6 hours with thrombin (not shown). An immunoblot anti-ERK1 and anti-ERK2 was performed at each time of activation to ensure that equal amounts of total ERKs were deposited on the gel before the blot analysis (Figure 2, bottom).

**ERK Phosphorylation and Localization Triggered by TRAP or Thrombin**

Phosphorylation of MAPKs is essential for their activation and nuclear translocation.\textsuperscript{11,13} Moreover, MAPK nuclear retention duration is critical for the determination of the response.\textsuperscript{14} We therefore studied the intracellular localization by immunofluorescence (Figure 3). For this purpose, the pERK1 and pERK2 were visualized by Texas Red, and the nucleus was visualized by coloring with blue. Before the addition of the inducers (Figure 3A), pERKs were at basal level and could not be detected at a significant level in the cytoplasm nor in the nucleus. After the addition of TRAP and thrombin, the two proteins were rapidly phosphorylated to the same level with the two agonists after 15 minutes (Figures 3C and 3D) and the pERKs were scattered in both the nucleus and the cytosol, with an important amount in the nucleus. After 4 hours of activation, ERKs were less phosphorylated than at short times and more retained in the nucleus of HUVECs with thrombin (Figure 3F) than with TRAP (Figure 3E). Thus, 27% of thrombin-stimulated cells exhibited high-level pERKs in their nucleus (Figure 3F) compared with 7% in TRAP-stimulated cells (\(P<0.04\)). The results suggest that in HUVECs the amount of DNA synthesis generated by PAR1 stimulation is inversely correlated with the duration of nuclear pERK retention.

**Regulation of the PAR1-Generated Activation Signal by Thrombomodulin**

In view of the modulating effect of TM on PAR1 activation that we described on DNA synthesis in HUVECs,\textsuperscript{9} we searched for a modulating effect of TM on ERK phosphorylation duration. For this purpose, we used 2 different tools: S195A, a mutant thrombin that binds to TM and has no proteolytic activity,\textsuperscript{18} and a specific anti-TM antibody. Results obtained with the mutant thrombin S195A are presented in Figure 4. The thrombin mutant S195A (200 nmol/L), which does not stimulate DNA synthesis,\textsuperscript{9} induced a low phosphorylation of ERKs (Figure 4B) compared with control (Figure 4A). The addition of S195A simultaneously with TRAP resulted in a higher ERK phosphorylation after 4 hours (Figure 4D) than in the presence of TRAP alone (Figure 4C). Moreover, S195A retained the pERKs in the nucleus (Figure 4D), whereas phospho-ERKs were no longer present after 4 hours of stimulation by TRAP alone (Figure 4C). The number of cells exhibiting a high level of pERK was increased from 7% with TRAP alone to 18% when S195A was added to TRAP.

We next prevented thrombin binding to TM with the monoclonal antibody 3E2 that we previously described to increase thrombin-induced DNA synthesis.\textsuperscript{9} Compared with control (Figure 5A), anti-TM by itself induced a slight
increase of ERK phosphorylation (Figure 5B), as did, however, an irrelevant antibody (Figure 5C). After 4 hours of stimulation by thrombin, the amount of pERKs in the nucleus (Figure 5D) was decreased in the presence of anti-TM (Figure 5E), suggesting that it accelerated the dephosphorylation. Moreover, in the presence of anti-TM, in 30% of the cells the pERKs remained at the edge of the nucleus membrane (red annulus around the blue nucleus) within the cytoplasm (Figure 5E) compared with 3% in the absence of the antibody. Thus, the number of thrombin-stimulated cells exhibiting a high level of pERKs in their nucleus was significantly reduced from 27% to 14% in the presence of the anti-TM antibody (P<0.02). These results suggest that thrombin binding to TM contributes to the maintenance of pERKs and nuclear retention.

Discussion

The activation of ERKs is an important step in cell stimulus–response coupling, and their localization is determinant of their function. In the present study, we found that after stimulation of HUVECs by thrombin or TRAP, ERK1 and ERK2 phosphorylation duration and nuclear retention were inversely correlated to the DNA synthesis. TRAP, a strong mitogen for HUVECs, induced a short-lasting phosphorylation and nuclear retention of pERKs; thrombin, which induced a lower DNA synthesis than did TRAP, induced a long-lasting phosphorylation and nuclear retention of pERKs. These results additionally demonstrate the regulatory role of TM in thrombin-induced PAR1 activation: TM, known to reduce HUVEC proliferation in response to thrombin, extended pERK nuclear retention.7,8

The activation of ERKs is dependent on a dual-specificity MAPK kinase, MEK.14,23 ERKs are phosphorylated on a TXY motif, both on threonine and tyrosine residues, and these phosphorylations are essential for activity.11 The stimulation of HUVECs by thrombin and TRAP led to DNA synthesis through activation of the ERK pathway. First, proliferation of DNA synthesis was strongly and dose-dependently inhibited by PD98059. PD98059 is a semiselective inhibitor of MEK1 and MEK2 and thus inhibits the phosphorylation of MEK substrates, the MAPKs. Second, thrombin and TRAP induced the phosphorylation of 2 members of the MAPK family, p42ERK1 and p42ERK2. This phosphorylation was rapid and reached a plateau of approximately similar amplitude with thrombin and TRAP. After longer
Phosphorylation and activation of transcriptional factor. In Bar305
The corresponding Western blots obtained with the anti–body 3E2 (E) for 4 hours before fixation and examination as in Figure 3. Unstimulated cells were incubated with an irrelevant antibody (B) or the anti-TM alone (C) and stained as in panel A. The corresponding Western blots obtained with the anti-phospho-ERK antibody as in Figure 2 are shown at the bottom. Bar=30 μm.

times (>2 hours), phosphorylation decreased, and the dephosphorylation was more rapid with TRAP than with thrombin. The duration of ERK phosphorylation and activation is critical for the cell signaling decision: cells use transient or sustained activation of ERKs, depending on the response. In the present study, we showed that in HUVECs, activation of PAR1 induced transient ERK phosphorylation and led to DNA synthesis.

The MAPK pathway that ultimately leads to cell proliferation or differentiation provides a mechanism that transmits the information to the nucleus. The nuclear translocation step concerns activated pERKs. Once in the nucleus, ERKs phosphorylate and activate transcriptional factor. In HUVECs, using immunocytochemical analysis, we showed that pERKs were translocated in the nucleus after stimulation by TRAP and by thrombin. However, the retention time in the nucleus was different with the two agonists. After 4 hours of stimulation, pERKs were still present in the nucleus of thrombin-activated cells, whereas they were barely detectable in TRAP-stimulated cells. Thus, pERK retention in the nucleus correlated with the protein phosphorylation state.

Thrombin and TRAP may activate different PAR receptors. At least 3 PAR receptors have already been described on endothelial cells (PAR1, PAR2, and PAR3), of which PAR1 and PAR2 are involved in endothelial cell proliferation. Moreover, TRAP is able to activate PAR1 even after thrombin cleavage. On the other hand, HUVEC proliferation induced by thrombin is downregulated by thrombin binding to TM. Overexpression of TM on smooth muscle cell surface decreases the TRAP-induced proliferation. Surface TM expression downregulates melanoma cell proliferation independently of its thrombin cofactor activity. In the strategy to specifically isolate the effect of thrombin binding to TM, we used a thrombin analogue devoid of any catalytic activity, S195A, and a specific monoclonal anti-TM antibody, 3E2. Hence, using these 2 different tools, we demonstrated that TM regulated the pERK nuclear retention. First, in the presence of anti-TM antibody that inhibits thrombin binding to TM, pERK nuclear retention was reduced. Second, the addition of an inactive thrombin mutant that binds to TM together with TRAP prolonged pERK nuclear retention. These data suggest that thrombin binding to TM triggers a signal that enhances pERK nuclear retention. Thus, in addition to its role of cofactor to activate protein C, TM seems to act as a new cell receptor for thrombin.

The mechanism required for the nuclear translocation has been elucidated for ERK2: phosphorylation creates charge-charge interactions that result in ERK dimerization, and dimers are actively imported into the nucleus. The transient phosphorylation of ERKs in stimulated HUVECs suggests that the proteins are under the control of phosphatases. Many cytosolic and nuclear MAPK phosphatases dephosphorylate ERKs in their respective cell compartments. Similar phosphatase-controlled phosphorylation regulation could occur in HUVECs. The results described above also suggest that TM could govern nuclear pERK retention. Thrombomodulin could trigger a signal to inhibit a nuclear MAPK phosphatase (MKP), such as MKP1 (Figure 6). MKP1 is implicated in the initiation of the cell cycle and activated by pERKs via a feedback control. It is not unlikely that TM controls MKP1 activation by pERKs.

Thrombin-induced endothelial cell response is of special importance during ischemia consecutive to arterial embolic disorders. The latter corresponds to pathological situations in which the endothelium integrity is relatively preserved, in contrast to local thrombosis occurring at the site of an atherosclerotic plaque rupture. The thrombin generated by an embolic clot comes into contact with endothelial cells and enhances edema, cell proliferation, and fibrosis. The involvement of TM, on the one hand, and pERK signaling, on the other hand, has been emphasized in arterial thrombotic disorders. ERK activation leads to deleterious effects during focal brain or myocardial ischemia. Although myocardial infarction is most frequently the consequence of local thrombosis, ischemic strokes more frequently result from embolic disorders. TM expression has been demonstrated in brain vessels, because TM mRNA expression is present in the blood-brain barrier and protein C is activated in jugular vein blood during carotid artery occlusion in humans. The endothelial TM expression in brain is constitutively downregulated by astrocytes and acutely downregulated by pathogenic mediators of ischemic stroke, such as interleukin-1β and tumor necrosis factor-α. Interestingly, astrocyte-induced downregulation of TM endothelial expression occurs through transforming growth factor-β secretion, known to enhance endothelial cell proliferation. Overall, that TM
expression and cell proliferation are inversely related fits with our results, showing a control of DNA synthesis by TM.

In conclusion, we show that TM controls the thrombin-induced ERK pathway in endothelial cells. Thus, TM, which plays a key role in the regulation of blood coagulation, could also be critically involved in the endothelial response to thrombosis. The latter would be of important clinical relevance in acute arterial ischemia, as recently suggested by epidemiological studies on myocardial infarction.39,40 It is thus worthwhile to study the implication of TM in ischemic stroke.

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