Thrombin mediates mitogenesis and survival of human endothelial cells through distinct mechanisms

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Zania P, Papaconstantinou M, Flordellis CS, Maragoudakis ME, Tsopanoglou NE. Thrombin mediates mitogenesis and survival of human endothelial cells through distinct mechanisms. *Am J Physiol Cell Physiol* 294: C1215–C1226, 2008. First published March 26, 2008; doi:10.1152/ajpcell.00452.2007.—Thrombin has been reported to interact with and stimulate normal and abnormal angiogenesis (40). The critical role of thrombin in the development of the vascular system was documented in mouse genetic models, where the lack of thrombin generation resulted in embryonic lethality and/or severe vascular defects during embryonic development (22). Thrombin has been reported to interact with and stimulate vascular cells, but the precise role and mode of action of thrombin in regulating the vascular formation has yet to be defined. Several vascular regulatory proteins and growth factors have been shown to be activated or upregulated by thrombin, suggesting an indirect action of thrombin in the regulation of blood vessel formation. For example, thrombin-induced angiogenesis in the chick chorioallantoic membrane system was associated with upregulation of the major vascular endothelial growth factor (VEGF) as well as angiopoietin-2 (Ang-2) (3). Thrombin upregulated VEGF in several cell types (13) and increased mRNA and protein levels for the VEGFR-2 in endothelial cells (38). In addition, thrombin upregulated Ang-2 (12) and activated metalloproteinase-2 (MMP-2) in endothelial cells (46) and induced the secretion of VEGF (21) and Ang-1 (16) from platelets. Thrombin also rapidly stimulated the expression and release of fibroblast growth factor-1 (FGF-1) in fibroblasts (4).

On the other hand, thrombin has been shown to directly promote endothelial cell mitogenesis. Several studies have provided evidence that thrombin-induced DNA synthesis is mediated by proteinase-activated receptor 1 (PAR1) and involves the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) in endothelial cells (24). However, the precise cellular mechanisms involved and the actual contribution of the mitogenic activity in thrombin-induced angiogenesis have not been yet elucidated.

A growing body of evidence points to the fact that thrombin can be pro- or anti-apoptotic in a variety of cell types, including epithelial and neuronal cells, fibroblasts, and tumor cells (7). Activation of PAR1, the principle thrombin receptor, has been found to induce or inhibit apoptosis, depending on the dosage of thrombin or that of the synthetic thrombin receptor activators (TRAPs). It is of interest that the effect of thrombin on endothelial cell apoptosis has not been reported thus far. Such an effect could be of great importance because thrombin is dramatically upregulated at sites of vascular injury, inflammation, or within the tumor microenvironment (23). In the present study, it is shown that the mitogenic effect of thrombin is much less pronounced compared with VEGF, basic FGF (bFGF), or epidermal growth factor (EGF) and is mediated indirectly by MMP-dependent release of heparin-binding epidermal growth factor (HB-EGF), transactivation of the EGF receptor (EGFR), and subsequent activation of ERK1/2. The PAR1 signaling to ERK1/2 is dependent on integrin-mediated anchorage to specific extracellular matrix proteins. We also demonstrate that thrombin potently protects endothelial cells from serum starvation-induced apoptosis via a mechanism in which its catalytic active site and PAR1 activation have limited involvement. The integrins αβ3 and αβ1 play an essential role in thrombin-induced cytoprotection. These results provide a better understanding of the mechanisms involved in thrombin-induced angiogenesis and point to the significant role of thrombin in regulating vascular functions.

**METHODS**

Reagents, antibodies, and cell cultures. Human thrombin (specific activity: 3093 IU/mg), PD-98059, AG-1478, LY-294002, [Glu32]diptheria toxin (CRM197), echistatin, fibronectin, collagen I, and poly-L-lysine were obtained from Sigma (St. Louis, MO). DIP-thrombin (thrombin that is chemically inactivated at the active site by disoprop-
and the acid-insoluble fractions were lysed in 0.5 N NaOH. The cells were then fixed and washed twice with 5% trichloroacetic acid, containing 0.5% BSA for 18 h. All cells were pulsed with 0.5 growth factors or inhibitors and antagonists in serum-free medium FBS for 24 h, cells were treated with the indicated concentration of confluency in 24-well plates. After the medium was changed to 4% both the 116-kDa intact and 85-kDa cleaved forms of PARP, anti-antibodies using the following concentrations: mouse anti-bcl-2 10% SDS-polyacrylamide gel electrophoresis and transferred to lyzed with RIPA lysis buffer, and pooled lysates were resolved with 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies using the following antibody concentrations: anti-phospho p42/44 mitogen-activated protein kinases (MAPK, ERK1/2) (1:3,000, New England Biolabs), which detects p42 and p44 MAPK only when catalytically activated by phosphorylation at Thr202 and Tyr204, anti-p42/44 MAPK (1:3,000, New England Biolabs), anti-phospho-EGFR (Tyr1068), which detects endogenous levels of EGFR when phosphorylated at Tyr1068 (1:1,000) and anti-α-tubulin (1:20,000, Sigma). Membranes were then probed with secondary antibodies conjugated with horseradish peroxidase and proteins were visualized by chemiluminescent detection.

For adhesion experiments, 60-mm tissue culture plates were coated with BSA or indicated proteins for 4 h at 37°C. Plates were then washed twice with PBS and blocked with 2% BSA for 1 h at 37°C. Serum-starved confluent HUVECs were dissociated with PBS-EDTA 1% and replated on protein-coated dishes at a cell density of 50–70%. After 3 h of incubation, cells were washed and stimulated for 10 min with the vehicle or the indicated agents. Attached cells were lysed and processed for ERK1/2 phosphorylation levels as described above.

For analysis of bcl-2 and poly(ADP-ribose) polymerase (PARP), endothelial cells were cultured in gelatin-coated 60-mm tissue culture plates. After reaching 70–80% confluency, cells were treated with thrombin for 24 h or staurosporine for 10 h. Attached and suspended cells were lysed with RIPA lysis buffer, and pooled lysates were resolved with 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies using the following concentrations: mouse anti-bcl-2 monoclonal (1:1,000; Upstate, Lake Placid, NY), mouse anti-PARP monoclonal (1:3,000; BD Biosciences, San Jose, CA), which detects both the 116-kDa intact and 85-kDa cleaved forms of PARP, anti-α-tubulin monoclonal (1:2,000; Sigma). [3H]thymidine incorporation assay. HUVECs were incubated until confluency in 24-well plates. After the medium was changed to 4% FBS for 24 h, cells were treated with the indicated concentration of growth factors or inhibitors and antagonists in serum-free medium containing 0.5% BSA for 18 h. All cells were pulsed with 0.5 μCi/ml [3H]thymidine (ICN Biomedicals, Irvine, CA) for an additional 6 h. Cells were then fixed and washed twice with 5% trichloroacetic acid, and the acid-insoluble fractions were lysed in 0.5 N NaOH. The radioactivity was determined in a liquid scintillation counter. Each experiment included three wells in each condition tested and was repeated at least twice. Results are expressed as means ± SE of disintegrations per minute per well and presented as percentage change of control (0%). Statistical analysis was performed with Student’s t-test.

Cell proliferation assay. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, endothelial cells (25,000 cells/well) were seeded in 24-well tissue culture plates and incubated with growth medium for 24 h. Cells were then treated with the vehicle or the indicated agents in serum-free medium containing 0.5% BSA. After 24 h, MTT (50 μl) solution (5 mg/ml) was added to each well and incubated for 3 h at 37°C. The blue formazan crystals were solubilized by the addition of DMSO (200 μl). Absorbance at 450 nm was recorded by using a 96-well plate reader. Experiments were run in triplicate and repeated at least twice. Results are expressed as means ± SE of optical density at 450 nm (OD450).

Statistical analysis was performed with Student’s t-test.

Cell cycle analysis. HUVECs were grown in 100-mm tissue culture plates until ~80% confluency. Endothelial cells were then washed and treated in the absence or in the presence of the indicated factors for 24 h in serum-free medium containing 0.5% BSA. After treatment, attached cells were collected by trypsinization, pooled with suspended cells, and washed and fixed in methanol for 1 h at −20°C. Fixed cells were then incubated with RNase (100 μg/ml, Sigma) for 30 min at 37°C and stained with propidium iodide (PI, 50 μg/ml, Sigma) for 20 min at 4°C in the dark. Flow cytometry was performed on a FACScan flow cytometer (EPICS XL-MCL; Coulter). The PI-stained cell population in sub-G0/G1, G1, S, and G2/M phases were represented by distinct and quantified peaks in the fluorescence histograms obtained using the WinMDI cell program. Results are expressed as mean percentage change of control ± SE. Experiments were run in triplicate and repeated twice. Statistical analysis was performed with Student’s t-test.

Assessment of apoptosis by flow cytometry analysis. To assess cell death specific to apoptosis, we used the annexin V-FITC assay kit (BD Biosciences Pharmingen). Endothelial cells were grown until ~80% confluency. Cells were then washed and treated in the presence or in the presence of the indicated growth factor or antagonists and inhibitors for 24 h in serum-free medium containing 0.5% BSA. After treatment, attached cells were collected by trypsinization, pooled with suspended cells, and washed in PBS. Cells (1×10⁶) were then resuspended in 100 μl of the kit reaction buffer containing PI and annexin V-FITC, according to the manufacturer’s instructions. After mixing was completed, cells were incubated for 15 min in the dark at room temperature and analyzed on the FACS flow cytometer within 1 h after staining. Cells were analyzed for healthy cells (annexin V- and PI-negative), early apoptotic cells (annexin V-positive, PI-negative), and late apoptotic or dead cells (annexin V- and PI-positive) by flow cytometry. The corresponding percentages of stained cells are shown in representative dot plots or expressed as mean change of control ± SE. Statistical analysis was performed for the early apoptotic cell population with Student’s t-test.

Caspase-3 activity assay. To examine the activity of caspase-3 in treated endothelial cells, it was used in an assay kit (Promega, Madison, WI). The colorimetric substrate, Ac-DEVD-p-nitroanilide, which was cleaved by caspase-3 to release yellow p-nitroanilide, was measured by absorbance at 405 nm. HUVECs were grown in 60-mm tissue culture plates until ~80% confluency. Endothelial cells were then washed and treated in absence or in presence of the indicated factors for 24 h in serum-free medium containing 0.5% BSA. Suspended cells as well as adherent cells were collected, washed, and lysed. Protein extracts were prepared and caspase-3 activity was measured following the manufacturer’s instructions. Absorbance at 405 nm was recorded using a 96-well plate reader. Experiments were run in triplicate and repeated twice. Results are expressed as means ± SE of optical density at 405 nm (OD405). Statistical analysis was performed with Student’s t-test.
RESULTS

Thrombin-induced mitogenesis is mediated by PAR1 activation and involves ERK1/2 activation. Consistent with previously published results (24), we show in this study that thrombin was capable of inducing $[^{3}H]$thymidine incorporation in endothelial cells by 97% compared with control (Fig. 1). Thrombin-induced DNA synthesis was PAR1 dependent and involved the phosphorylation of ERK1/2 in HUVECs, since the agonist peptide TRAP-1 (SFFLRN) also increased endothelial cell DNA synthesis (107% over that of control) and PD-98059, a selective and cell-permeable inhibitor of MAPK kinase (MEK), completely reversed the stimulating effect of thrombin (Fig. 1). The phosphorylation of ERKs was analyzed by Western blot analysis by using an antibody specific for the phosphorylated forms of p44ERK1 and p42ERK2. After the addition of thrombin or TRAP-1, the two proteins were rapidly phosphorylated after 10 min, whereas the addition of DIP-thrombin (the catalytically inactive thrombin) did not exhibit any effect on ERK1/2 activation (Fig. 2A). When thrombin was preincubated with hirudin, a potent and selective thrombin inhibitor, the effect of thrombin was cancelled out, thus establishing the specificity of thrombin action (Fig. 2A). The fact that thrombin-induced ERK1/2 activation was exclusively mediated by PAR1 activation was further established by the use of the PAR1 cleavage-blocking monoclonal antibodies WEDE15 and ATAP12 (6) and the selective PAR1 antagonist SCH-79797 (15), which both completely abolished the activation of ERK1/2 by thrombin (Fig. 2A).

Thrombin-induced mitogenesis is mediated through EGFR transactivation in endothelial cells. To investigate the potential role of EGFR signaling in thrombin-induced ERK1/2 activation and mitogenesis in endothelial cells, we treated HUVECs with tyrphostin AG1478, which is an EGFR tyrosine kinase-specific inhibitor, before stimulation with thrombin. Thrombin-stimulating DNA synthesis and ERK1/2 activation were completely inhibited by AG1478 (Figs. 1 and 2B). Similar results were obtained in TRAP-1-stimulated endothelial cells. Under the same conditions the stimulation of endothelial cells by EGF or HB-EGF was also completely abrogated by AG1478, but the stimulation by bFGF was not affected. In addition, we assayed endothelial cells for tyrosine-specific phosphorylation of the EGFR by thrombin. The EGFR became highly phosphorylated in response to EGF and HB-EGF stimulation, whereas thrombin treatment resulted in moderate EGFR tyrosine phosphorylation (Fig. 2C). These results point to a stringent requirement of EGFR kinase activity in the PAR1 signaling to ERK1/2.

We next investigated whether the EGFR transactivation observed upon PAR1 activation is mediated by HB-EGF release, which together with neuregulin-1 are the only members of the EGFR family ligands found in HUVECs (personal observations and 14). We analyzed the involvement of HB-EGF by blocking its biological activity with a neutralizing antibody or using CRM197, a specific inhibitor of HB-EGF. As shown in Figs. 1 and 2D, treatment of endothelial cells with these two agents completely abolished thrombin-induced ERK1/2 activation and DNA synthesis. Similar inhibition, as expected, was also observed for HB-EGF. In contrast, EGF-induced activation was unaltered by CRM197 and neutralizing antibody, thus demonstrating their specificity. These findings suggest that HB-EGF mediated EGFR transactivation upon activation of PAR1 in HUVECs.

The potential role of metalloproteases (MMPs) in thrombin-induced mitogenesis was investigated, because MMPs have been implicated in pro-HB-EGF shedding (27). Pretreatment of endothelial cells with the general MMPs inhibitor GM-6001 showed that $[^{3}H]$thymidine incorporation as well as activation of the ERK1/2 in response to thrombin was abolished (Figs. 1 and 2E). The inhibition was specific because GM-6001 was without effect when HUVECs were stimulated with EGF, bFGF, VEGF, or HB-EGF. In addition, the fact that GM-6001 inhibited TRAP-1-induced mitogenesis ruled out the possibility that it might affect the proteolytic activity of thrombin. In an attempt to identify distinct proteolytic enzymes involved in thrombin-mediated EGFR transactivation, we initially focused on MMP-2 and MMP-9. These two MMPs are expressed in human endothelial cells and MMP-2 has been shown to be activated by thrombin (46). We used two potent inhibitors for MMP-2 and MMP-9, inhibitor II and inhibitor III. As shown in Fig. 2E, both MMP-2/MMP-9 inhibitors were without effect on thrombin-induced ERK1/2 activation even at high concentrations, indicating that MMP-2 and MMP-9 were unlikely to be involved in thrombin-induced cleavage and release of HB-EGF. We also tested the possible involvement of a disintegrin family of proteases (ADAMs) in ERK1/2 activation by thrombin. Experiments with small interfering RNAs that specifically downregulated the ADAM9, ADAM10, ADAM15, and ADAM17 mRNA levels, revealed that these ADAMs were not involved in the cross-talk between the PAR1 and the EGFR in HUVECs (data not shown).

PAR1 activation of ERK1/2 is dependent on integrin-mediated anchorage. Earlier studies have shown that efficient signal transduction from G protein-coupled receptors to MAPK requires integrin-mediated cell anchorage (33). HUVECs display several integrins on their surface, including $\alpha_{5}\beta_{1}$, $\alpha_{v}\beta_{3}$, and $\alpha_{v}\beta_{1}$, which are important receptors for fibronectin, vitronectin and collage I, respectively (34). To evaluate the role of integrin-mediated cell anchorage in signaling to ERK1/2 from

Fig. 1. Thrombin (Thr)-induced mitogenesis in endothelial cells is mediated by proteinase-activated receptor (PAR1) and involves extracellular signal-regulated protein 1/2 (ERK1/2) activation. Growth factor-starved human umbilical vein endothelial cells (HUVECs) were incubated in serum-free medium in presence of vehicle (control), Thr (3 IU/ml), TRAP-1 (100 $\mu$M), PD-98059 (PD, 30 $\mu$M), AG-1478 (AG, 500 nM), CRM-197 (CRM, 10 $\mu$g/ml), GM-6001 (GM, 10 $\mu$M), EGF (10 ng/ml), HB-EGF (HB, 10 ng/ml), bFGF (5 ng/ml) or the indicated combinations for 18 h. All cells were pulsed with $[^{3}H]$thymidine for an additional 6 h. Each experiment included three wells in each condition tested and was repeated at least twice. Results are expressed as means ± SE of disintegrations per minute per well and presented as percentage change of control (0%). Statistical analysis was performed with Student’s t-test. *$P < 0.01$. 

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Fig. 2. Mechanism of Thr-induced ERK1/2 activation in endothelial cells. A, B, D, and E: serum-starved HUVECs were treated for 10 min with vehicle (C, control), Thr (3 IU/ml), hirudin (Hir, 10 IU/ml), or the combination of WEDE15 and ATAP12 antibodies (WEDE/ATAP, 20 μg/ml each) or SCH-79797 (SCH, 1 μM), TRAP-1 (100 μM), DIP-thrombin (DIP-Thr, 5 μg/ml), EGF (10 ng/ml), heparin-binding-EGF (HB, 50 ng/ml), AG-1478 (AG, 500 nM), bFGF (5 ng/ml), GM-6001 (GM, 10 μmol/l), or VEGF (10 ng/ml). In combination experiments, cells pretreated for 15–30 min with vehicle or WEDE15/ATAP12 antibodies, SCH-79797, AG-1478, or anti-HB-EGF neutralizing antibody (a-HB, 50 μg/ml), CRM-197 (CRM, 10 μg/ml), GM-60001, MMP2/MMP9 inhibitor II (Inh II, 300 nM), or MMP2/MMP9 inhibitor III (Inh III, 100 μM). Cell lysates were probed with anti-phospho-ERK1/2-specific antibody. To determine total protein level, membranes were probed with ERK1/2 antibody. C: quiescent cells were treated with vehicle (C, control) or stimulated with Thr (3 IU/ml), heparin-binding EGF (10 ng/ml), or EGF (10 ng/ml) for 3 min, and the specific phosphorylation of EGFR in Tyr1068 was detected by immunoblotting. To ensure loading of equal protein amounts, the membrane was re-probed with anti-α-tubulin antibody. All experiments were performed independently at least three times. Representative blots are shown.
PAR1 receptors, serum-starved HUVECs were either maintained in suspension or allowed to adhere to different proteins before stimulation with thrombin. HUVECs attached and spread on these extracellular matrix proteins almost to the same extent. As shown in Fig. 3, ERK1/2 was activated in a specific way in cells that were adherent to substrata coated with fibronectin and vitronectin but not in collagen I. In addition, cells maintained in suspension or cells nonspecifically adherent to substrata coated with poly-L-lysine showed virtually no activation. There was no indication of a dose-responsive activation of ERK1/2 in cells held in suspension (data not shown). Moreover, the loss of ERK1/2 activation by thrombin was not due to cell death or irreversible changes, since bFGF was able to activate ERK1/2 in suspended cells (Fig. 3), and when suspension cells were replated on fibronectin, they rapidly regained their ability to respond to thrombin by activation of ERK1/2 (data not shown). These findings indicate that efficient signal transduction from PAR1 to ERK1/2 requires specific integrin-mediated cell anchorage.

**Thrombin is a poor growth but a potent anti-apoptotic factor for endothelial cells.** As mentioned above, thrombin, through PAR1 activation, increased phosphorylation levels of ERK1/2 and subsequently activated mitogenesis in endothelial cells. However, this effect of thrombin was less pronounced compared with that of other growth factors such as EGF, HB-EGF, bFGF, or VEGF (Figs. 1 and 2). The question then arises if thrombin can be regarded as an effective growth factor for endothelial cells and thus can contribute substantially to endothelial cell growth. In experiments, where the growth rate of endothelial cells was estimated after 24 h, the presence of thrombin caused a nonsignificant increase on cell population (only 9%) when compared with the number of cells at the beginning of the experiments (Fig. 4A). Similar results were obtained by the use of TRAP-1. On the other hand, VEGF and HB-EGF, which caused much stronger activation of ERK1/2 phosphorylation and DNA synthesis, promoted endothelial cell proliferation by 31% and 18%, respectively (Fig. 4A). However, when we measured the number of endothelial cells in the presence or absence of thrombin for 24 h, the number of cells exposed to thrombin was significantly higher compared with cells without thrombin (Fig. 4A). These results provided the first indication that thrombin is a rather weak growth factor for endothelial cells, but it may have significant protective effects by inhibiting apoptosis.

Flow-cytometric cell cycle analysis was performed to further determine whether the results in growth experiments were a reflection of cell cycle progress (proliferating state) and/or survival. The sub-G0/G1 fraction (subdiploid region on the DNA content histogram) was used as a measure of the per-
centage of apoptotic cells, whereas the S/G2/M fraction was used as index of proliferating cells. As shown in Fig. 4B, thrombin caused a small (9%) induction of the percentage of endothelial cells in S/G2/M phase, whereas EGF, VEGF, and HB-EGF markedly increased the percentage of proliferating cells by 48, 50, and 38%, respectively. Thrombin, however, reduced substantially the apoptotic cell population by 28%, whereas EGF (12%) and HB-EGF (9%) caused moderate effects. VEGF exhibited the most pronounced effect reducing the apoptotic cells fraction by 52%. These results point to differing abilities of these factors to trigger survival and proliferation in endothelial cells.

From the above experimental data, we explored in detail the role of thrombin in serum deprivation-induced apoptosis of endothelial cells. We used the annexin V/PI-based assay, which is a valuable and very sensitive technique to detect apoptosis, and it is used by many investigators in a variety of settings (42). Cells, negative for both PI and annexin V staining, represent alive and healthy cells; PI-negative, annexin V-positive staining cells are early apoptotic cells; PI-positive, annexin V-positive staining cells are primarily cells in a late stage of apoptosis or dead. The data presented in Fig. 5A revealed that thrombin decreased the percentage of endothelial cells in the early and late apoptotic state in a concentration-dependent manner. In parallel, the percentage of healthy cells was equally increased. This effect was also evident after a longer (36 h) incubation time of endothelial cells (Fig. 5A). In addition, we compared the anti-apoptotic effect of thrombin with that of EGF, HB-EGF, bFGF, and VEGF. VEGF and bFGF reduced the apoptotic cell population by 63% and 59%, respectively, which were comparable with that obtained with thrombin (54%) (Fig. 5B). However, EGFR ligands caused a less pronounced anti-apoptotic effect. EGF and HB-EGF reduced the serum starvation-induced apoptosis of HUVECs with a bell-shaped, dose-response curve, with the maximal effect at 10 (28%) and 50 ng/ml (35%), respectively (Fig. 5B). Higher concentrations resulted in diminishing effects on apoptosis. Although the cause of this effect is not entirely clear, a current thought is that a vast excess of ligand over receptors results in a reduced effectiveness of receptor dimerization and/or in receptors downregulation (27). From these results, which are in agreement with those presented in Fig. 4B, we conclude that thrombin is a potent protection factor for endothelial cells comparable to bFGF and VEGF and greater than EGF and HB-EGF.

Fig. 5. Thr is a potent anti-apoptotic factor for endothelial cells. A: HUVECs were treated under serum-free conditions in the presence of vehicle (None, control) or the indicated concentration of Thr for 24 or 36 h. B: HUVECs were treated under serum-free conditions in the presence of vehicle (None, control) or Thr (3 IU/ml) or the indicated concentration of EGF, HB-EGF, or the combination of Thr (3 IU/ml) with EGF (100 ng/ml), HB-EGF (100 ng/ml), bFGF (10 ng/ml), or VEGF (50 ng/ml) for 24 h. Cells were analyzed for healthy cells (annexin V- and PI-negative), early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic, or dead cells (annexin V- and PI-positive) by flow cytometry. The corresponding percentages of stained cells are shown in representative dot plots (A) or expressed as mean change of control ±SE (B). Statistical analysis was performed in early apoptotic cell population with Student’s t-test. *P < 0.05 and **P < 0.01 compared with the untreated cells.
Thrombin protects endothelial cells from apoptosis through a mechanism mostly independent of its catalytic active site and PAR1 activation: involvement of a bcl-2-dependent mechanism. To investigate the mechanisms of thrombin-induced endothelial cell protection, we first tested the effect of agonist peptides TRAP-1 (SFLRKN) and TRAP-4 (GYPGQV) on serum deprivation-induced apoptosis of HUVECs. As shown in Fig. 6, TRAP-1 revealed a small (12%) but statistically significant anti-apoptotic effect, whereas TRAP-4 was without effect at any concentration used. The modest involvement of PAR1 to thrombin-induced endothelial cell protection was further established by the use of the combination of WEDE15/ATAP12 antibodies. PAR1 cleavage-inhibiting antibodies only partially inhibited the thrombin-induced anti-apoptotic effect (Fig. 6). Interestingly, the exposure of HUVECs to DIP-thrombin resulted in significant reduction of apoptotic cells by 35% when compared with control, which was abolished by hirudin (Fig. 6). In agreement with these findings, when endothelial cells were exposed to PD-98059, AG-1478, or GM-6001, thrombin retained its ability to reduce cell apoptosis (data not shown). In addition, when thrombin was combined with high concentrations (100 ng/ml) of EGF or HB-EGF, the anti-apoptotic effect of thrombin was not altered (Fig. 5B). These results suggest that the ERK1/2 and EGFR pathways were not involved in the anti-apoptotic action of thrombin. The above results also point to the limited involvement of catalytic active site and PAR1 activation in the anti-apoptotic effect of thrombin and indicate that thrombin regulates mitogenesis and survival of endothelial cells through distinct mechanisms.

From the important role of phosphoinositide-3 kinase (PI-3K) pathway in endothelial cell survival by growth factors (8, 10), we investigated the involvement of PI-3K in thrombin-mediated cytoprotection. We preincubated HUVECs with LY-294002, a specific inhibitor of PI-3K. As assessed by the numbers of apoptotic cells, LY-294002 did not affect the ability of thrombin to protect endothelial cell from apoptosis, whereas it almost completely blocked the protective effect of VEGF (Fig. 7A). LY-294002 enhanced, in a concentration-dependent manner, the degree of apoptosis observed in the absence of thrombin, possibly resulting from the inhibition of basal PI-3K activity present in serum-starved cells. These results suggest that the anti-apoptotic activity of thrombin on endothelial cells is not mediated via PI-3K.

To further investigate the anti-apoptotic signaling of thrombin, lysates of HUVECs, which had been treated for 24 h with thrombin or DIP-thrombin or TRAP-1, were subjected to Western blot analysis for bcl-2, a key regulator of apoptosis by the intrinsic (mitochondrial) pathway. These studies showed that both thrombin and DIP-thrombin increased the protein levels of bcl-2, whereas TRAP-1 was without significant effect (Fig. 7B). Again, PAR1 cleavage-inhibiting antibodies WEDE15/ATAP12 failed to abolish the bcl-2 protein levels induced by thrombin. These data suggested that the mitochondrial pathway was involved in the anti-apoptotic response to thrombin. To examine the downstream sequence of events, we examined the caspase-3 activation as well as PARP cleavage. Caspase-3 activity extracted from the cells was measured over 24 h. In these experiments, bFGF reduced sufficiently the level of caspase-3 activity, whereas thrombin did not cause any significant alteration (Fig. 7C). Similarly, thrombin did not affect PARP cleavage to its signature 85-kDa fragment (Fig. 7D). Staurosporine and bFGF were used as negative and positive control respectively.

Integrins αvβ3 and α5β1 are essential in thrombin-induced cytoprotection. Integrins have been implicated in endothelial cell apoptosis, and important interactions between thrombin and integrins αvβ3 and α5β1 have been described (25, 36, 37). To investigate the role of these integrins in thrombin-induced endothelial cell survival, we used echistatin, a member of the disintegrins family, which is a very potent antagonist of β3-and β1-integrin family. When echistatin, at as low concentrations as 1 nM, was combined with thrombin, the protective effect of thrombin was almost blocked (Fig. 8A). The remaining part of protective effect of thrombin can be attributed to the partial involvement of PAR-1 activation on the mechanism of thrombin-induced protection. This point is supported by the fact that echistatin was unable to inhibit TRAP-induced survival of endothelial cells, even at a concentration of 10 nM (Fig. 8B). Echistatin also abolished the anti-apoptotic effect of DIP-thrombin (Fig. 8A). The specific involvement of αvβ3 and α5β1 integrins was further demonstrated by the use of neutralizing monoclonal antibodies against αvβ3 (LM-609) and α5β1 (anti-α5β1) integrins. As shown in Fig. 8A, pretreatment of HUVECs with LM-609 alone resulted in marked inhibition of thrombin-induced protection. Moreover, the exposure of cells to the combination LM-609/anti-α5β1 blocked the protective effect of thrombin almost completely (Fig. 8A). Again, the remaining part of cell survival effect of thrombin can be attributed to the partial involvement of PAR1, since both...
neutralizing antibodies failed to inhibit the TRAP-1-mediated cytoprotection (data not shown).

To examine the specificity of the involvement of integrins in the cytoprotection induced by thrombin, we analyzed the effect of echistatin and LM-609 antibody in thrombin-induced ERK1/2 activation. At the concentrations used above, neither echistatin nor LM-609 antibody inhibited the stimulatory effect of thrombin in ERK1/2 phosphorylation levels (Fig. 8C). Similarly, echistatin was without effect in DNA synthesis triggered by thrombin (Fig. 8C). In addition, echistatin did not influence the cytoprotection induced by VEGF or bFGF (Fig. 8B). Our findings indicate that αβ3 and α5β1 integrins are critically involved in mediating PAR-1-independent effects of thrombin on endothelial cell survival.

DISCUSSION

The main new finding in this study is that thrombin is able to protect endothelial cells from apoptosis induced by serum deprivation via a cellular mechanism that is different from that of its mitogenic or angiogenic effects. Thrombin, through PAR1 signaling, interacts and stimulates a variety of vascular cells and regulates the release, expression, and activation of the majority of angiogenesis mediators. These factors in turn promote endothelial cell proliferation, migration, differentiation, and vessel spouting. Thrombin has also been proposed to activate the proliferation of endothelial cells by acting directly as a mitogenic factor. However, the results presented in this study demonstrate that the mitogenic effect of thrombin in HUVECs was mediated indirectly via HB-EGF release. We have shown that activation of PAR1 by thrombin or the agonist peptide TRAP-1 resulted in a series of events that included MMP-dependent release of HB-EGF, transactivation of EGFR, activation of ERK1/2, and subsequent activation of DNA synthesis in endothelial cells. Similar transactivation of the EGFR has been reported to be involved in response to thrombin stimulation of rat vascular smooth muscle cells (2) and fibroblasts (30), whereas in astrocytes, EGFR transactivation is not necessary for thrombin to induce ERK1/2 phosphorylation (43). In human smooth muscle cells, thrombin-induced DNA synthesis is mediated by transactivation of FGFR-1 (29). Under our experimental conditions, it was also shown that in
endothelial cells the mitogenic activity of thrombin was much less pronounced compared with that of EGF, bFGF, or VEGF. In addition, in cell growth experiments, thrombin failed to effectively increase the cell proliferation. These observations led us to conclude that thrombin should not be considered as a directly functioning growth factor for endothelial cells. Whether thrombin-mediated release of HB-EGF plays an important role in vascular biology remains to be established. It may be of significance that HB-EGF has been shown to have mitogenic and chemotactic effects on pericytes and smooth muscle cells (1, 14). This may link thrombin to late stages of angiogenesis where smooth muscle cells and pericytes are recruited.

On the other hand, our results showed for the first time a potent anti-apoptotic effect for thrombin on serum-starved endothelial cells. In contrast to its mitogenic effect, the thrombin-mediated cytoprotection was almost PAR1-independent and was comparable to that of VEGF and bFGF and superior to that of EGF and HB-EGF. In addition, our studies showed that the ERK1/2 and EGFR pathways were not involved in the anti-apoptotic effect of thrombin. A striking demonstration of the distinct mechanism of thrombin-induced cell survival was
obtained from experiments with DIP-thrombin. DIP-thrombin was mimicking to a significant extent the anti-apoptotic effect of thrombin itself in endothelial cells. This restricted the involvement of the catalytic site of thrombin and the requirement for proteolytic activation of thrombin receptors on endothelial cells. However, when thrombin was combined with the potent and highly selective inhibitor hirudin, its anti-apoptotic effect was abolished. Hirudin binds to thrombin at the femtomolar range and is an exceptional good probe of the conformational state of the enzyme because it covers 20% of its solvent exposed surface area (20). In particular, hirudin binding is extended from a catalytic active site of thrombin to anion-binding exosite, which is used by thrombin to interact with the majority of its substrates. It is therefore possible that hirudin abrogates the anti-apoptotic action of thrombin by preventing the interaction with the responsible binding sites on the endothelial cells. This was further supported by the fact that hirudin was also able to completely block the anti-apoptotic effect of DIP-thrombin.

Integrins have been shown to be involved in the regulation of thrombin’s effects on platelets and smooth muscle cells (36). In endothelial cells, however, integrins have been described to play an important role only in mediating cellular effects triggered by immobilized thrombin (39). It has been demonstrated recently that immobilized thrombin functions directly through its RGD sequence, interacts with αvβ3 and α5β1 integrins, and induces attachment and migration of endothelial cells (25). In the present study, it was shown that αvβ3 and α5β1 integrins are involved in mitogenic and anti-apoptotic effect of thrombin but in a different manner. In the case of the mitogenic effect of thrombin, integrin ligation was critical for supporting ERK1/2 activation. In suspended endothelial cells, thrombin failed to activate ERK1/2, and the engagement of specific endothelial cell integrins by fibronectin and vitronectin was required for activation of ERK1/2 by thrombin. Similar observations have been described in several recent reports, where it has been demonstrated that integrin-mediated cell anchorage can regulate the efficiency of signaling from G protein-coupled receptors to ERK1/2 (33). However, the precise mechanistic basis for the role of integrins in PAR1 signaling is not yet defined. A plausible mechanism is that PAR1 transactivates the EGF receptor, thus leading to stimulation of the canonical EGFR-Ras-MARK cascade. If that were so, then anchorage regulation of PAR1 signaling to ERK1/2 might simply be a recapitulation of the previously described anchorage regulation of EGFR signaling (34). Furthermore, our data provide evidence that αvβ3 and α5β1 integrins do not participate in PAR1 signaling cascade to ERK1/2, since treatment of attached endothelial cells with echistatin or monoclonal antibody against αvβ3 integrin (LM609) did not affect the ability of thrombin to promote ERK1/2 activation and DNA synthesis.

Interestingly, in the present study, it was shown that αvβ3 and α5β1 integrins have also an essential involvement in the activation of endothelial cell survival by thrombin. Our findings suggest, for the first time, that native thrombin in solution may interact with these integrins in a way that can be inhibited specifically by disintegrin echistatin and antibodies against αvβ3 and α5β1 integrins. This raises the possibility that exposure of the RGD sequence of thrombin may also occur independently of matrix attachment. In addition, from the evidence that PI-3K/bcl-2 pathway plays an important role in endothelial cell survival (35) and the activation of integrins confer resistance to exogenous proapoptotic agents by increasing the expression of the anti-apoptotic protein bcl-2 (26), we explored the relationship between these molecules and thrombin-induced survival. Our data suggest induction of bcl-2 is a critical mechanism underlying thrombin-mediated resistance to apoptosis in human endothelial cells. On the contrary, pretreatment of endothelial cells with LY-290042 did not have any effect on the protection of serum-deprived endothelial cell apoptosis by thrombin. Together these findings provide strong evidence that thrombin suppresses apoptotic signaling in endothelial cells by at least two mechanisms. A minor contribution is mediated by PAR1 activation and a major contribution by interaction with αvβ3 and α5β1 integrins, in which the catalytic site of thrombin is not essential. We cannot rule out, however, the possibility that thrombin may also interact with other members of the integrin family or with other receptors of thrombin on endothelial cells, such as thrombomodulin. We consider the cellular mechanisms responsible for anti-apoptotic effect of thrombin as well as the signal transduction mechanisms involved in these events of great importance and are of high priority in our investigations.

Furthermore, our observations provide novel insights on the potential role of thrombin in vascular protection and evidence for an essential contribution of thrombin in the establishment and maintenance of vessel wall integrity. Vascular protection can be considered as a distinct nonangiogenic process through which thrombin can enhance endothelial cell functions that lead to inhibition of vascular smooth muscle cell proliferation, endothelial cell survival, and suppression of thrombotic and inflammatory events. For example, a number of studies have indicated that thrombin-generated activated protein C (APC) can regulate endothelial cells survival and it has protective effects in systemic inflammation (5, 41). Also, thrombin increases endothelial expression of complement inhibitory proteins (e.g., decay accelerating factor) (17) and induces the production and release of nitric oxide and PGI2 in endothelial cells (19), which have several effects that may play vascular protective roles, including vasodilatory properties, the inhibition of endothelial and smooth muscle cell proliferation (9, 11, 32), antplatelet action (31, 44), antiapoptotic effect (18), and inhibition of leukocyte adhesion (15). Therefore, vascular protection may provide an attractive alternative mechanistic framework for understanding the impact of thrombin on the cardiovascular system.

We conclude from these studies that, unlike other angiogenic factors, thrombin through its multiplicity of effects on angiogenesis, survival, interaction with other factors, and many cell types, may have the unique ability to orchestrate the requirements for the formation of mature blood vessels and a such may have therapeutic potential applications.  

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Thrombin Protects Endothelial Cells from Apoptosis


