Thrombin Suppresses Endothelial Nitric Oxide Synthase and Upregulates Endothelin-Converting Enzyme-1 Expression by Distinct Pathways
Role of Rho/ROCK and Mitogen-Activated Protein Kinase

Masato Eto, Christine Barandiér, Lisa Rathgeb, Toshiyuki Kozai, Hana Joch, Zhihong Yang, Thomas F. Lüscher

Abstract—An imbalance of nitric oxide and endothelin plays an important role in cardiovascular disease. Thrombin exerts profound effects on endothelial function. The present study investigated the molecular mechanisms by which thrombin regulates endothelial nitric oxide synthase (eNOS) and endothelin-converting enzyme (ECE)-1 expression in human endothelial cells. Incubation of human umbilical vein endothelial cells with thrombin (0.01 to 4 U/mL) for 15 to 24 hours markedly downregulated eNOS and increased ECE-1 protein level in a dose-dependent manner. Thrombin also decreased eNOS mRNA and increased ECE-1 mRNA level. In mRNA stability assay, thrombin shortened the half-life of eNOS mRNA but not that of ECE-1 mRNA. Activation of protease-activated receptor 1 by the agonist (SFLLRN, 10 to 100 \( \mu \)mol/L) had no effect on eNOS expression but increased ECE-1 level as thrombin. Thrombin activated Rho A and extracellular signal–regulated kinase (ERK)1 and ERK2. Inhibition of Rho A by C3 exoenzyme (20 \( \mu \)g/mL) and ROCK by Y-27632 (10 \( \mu \)mol/L) prevented the downregulation of eNOS expression by thrombin. Y-27632 also prevented the reduction in NOS activity induced by prolonged incubation with thrombin. On the other hand, inhibition of ERK1 and ERK2 activation by PD98059 (50 \( \mu \)mol/L) prevented the upregulation of ECE-1 expression by thrombin as well as the increase in ECE activity and ET-1 accumulation in the medium. Treatment of rat aorta with thrombin overnight impaired endothelium-dependent relaxations but not endothelium-independent relaxations. Thus, thrombin suppresses eNOS and upregulates ECE-1 expression via Rho/ROCK and ERK pathway, respectively. These effects of thrombin may be important for endothelial dysfunction in cardiovascular disease, particularly during acute coronary episodes. (Circ Res. 2001;89:583-590.)

Key Words: cell signaling • mitogen-activated protein kinase • endothelial dysfunction • protease-activated receptor

Atherosclerosis is the leading cause of death and accounts for half of the morbidity and mortality in Western countries. An imbalance of endothelium-derived relaxing and contracting factors is a hallmark of cardiovascular disease. Indeed, in human atherosclerosis, the production of nitric oxide (NO) is decreased because of reduced endothelial NO synthase (eNOS) expression. On the other hand, endothelin-1 (ET-1) production and endothelin-converting enzyme-1 (ECE-1) expression are increased in atherosclerosis and restenosis. However, the risk factors or mechanisms that lead to this imbalance of endothelial function in human atherosclerosis have not been completely elucidated.

Thrombin, the multifunctional enzyme generated in the context of vascular injury from the circulating zymogen prothrombin, is focused in atherosclerosis and its complications. Thrombin plays an important role in platelet activation, modulation of vasomotion, and vascular smooth muscle proliferation or migration and in turn contributes to vasospasm and vascular remodeling. Acutely, thrombin stimulates eNOS activity and releases NO or prostacyclin from endothelial cells, which both antagonize contraction and platelet aggregation. This may represent an important feedback mechanism to prevent vascular occlusion. However, the prolonged effect of thrombin on eNOS expression is unknown. Moreover, thrombin is also a potent stimulator of endothelial ET-1 production. ET-1 is produced from preproET-1, cleaved by a furin protease to big ET-1 and additionally processed to bioactive ET-1 by ECE-1. ECE-1 expression is increased in atherosclerosis and restenosis.
suggesting a potential role in vascular disease. However, the effect of thrombin on ECE-1 expression is unknown.

Much effort has been made to delineate molecular mechanisms by which thrombin affects cellular function. Thrombin exerts its cellular effect by activation of G protein–coupled protease-activated receptors (PARs). Four such receptors have been identified and cloned; three of these (PAR1, PAR3, and PAR4) are activated by thrombin. PAR1 is the prototype thrombin receptor and is activated when thrombin cleaves its NH₂-terminal exodomain to unmask a new receptor NH₂ terminus, which then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling. It seems that PAR3 and PAR4 mediate thrombin’s effect of mouse platelets, and PAR1 and PAR4 mediate activation of human platelets. An array of intracellular signal transduction pathways are activated by thrombin, such as phospholipase A₂, protein kinase C, phosphoinositol-3 kinase, extracellular signal–regulated kinases (ERKs), S6 kinase, and Rho in different cell types. The small GTP-binding protein Rho plays critical roles in gene expression, cell growth, migration, and contraction by binding to and activating several downstream effectors, such as ROCK/Rho kinases, protein kinase C–related protein kinases, and Citron kinase.

In the present study, we investigated the effects of thrombin on eNOS and ECE-1 expression and the underlying intracellular signaling mechanisms in cultured human endothelial cells.

### Materials and Methods

#### Materials and Chemicals

All cell culture were from Gibco BRL. Human thrombin was purchased from Sigma. *Clostridium botulinum* C3 exoenzyme and thrombin receptor agonist peptide (TRAP. SFLRN) were from Calbiochem. PD98059 and mouse monoclonal antibody against phospho-ERK1 and -ERK2 were from New England Bio-Labs (Allschwil, Switzerland). Rabbit polyclonal antibodies against RhoA (Sc-119) were purchased from Santa Cruz Biotecology (Basel, Switzerland). Mouse monoclonal antibody against human eNOS (N3020) was from Transduction Laboratories (Basel, Switzerland). Mouse monoclonal antibody against ECE-1 was kindly provided by K. Shimada and K. Tanzawa (Sankyo, Tokyo, Japan). The ROCK inhibitor Y27632 was kindly provided by Welfide Corporation (Osaka, Japan).

#### Cell Culture

Endothelial cells were isolated from human umbilical veins (HUVECs), as described. Briefly, fresh blood vessels were harvested in cold sterile RPMI 1640 medium with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The vessels, cleaned of connective tissue and adventitia, were incubated with 75 U/mL collagenase type II for 15 minutes in PBS. Cell pellets were then collected by centrifugation at 1000 rpm for 10 minutes and seeded in culture dishes coated with 25 μg/mL human fibronectin and cultured in RPMI 1640 supplemented with 20 mmol/L l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, 25 μg/mL heparin, and 20% FCS. The next day, cells were washed with the medium to eliminate blood cells. Endothelial cells were characterized by typical cobblestone and nonoverlapping appearance and indirect immunofluorescence staining using specific antibody against von Willebrand factor. Cells of 2nd to 3rd passage were used.

#### Endothelial NOS and ECE-1 mRNA Expression

Confluent endothelial cells were rendered quiescent for 24 hours by changing the medium to RPMI 1640 with the same ingredients as described above except that endothelial cell growth supplement and heparin were avoided and only 0.5% FCS was added. To study the effects of inhibitors of signal transduction pathways, the cells were stimulated with thrombin (4 U/mL) or TRAP (10 to 100 μmol/L) for the indicated time in the presence or absence of specific inhibitors and then washed twice with PBS, harvested in the extraction buffer (in mmol, sodium chloride 120, Tris 50, sodium fluoride 20, benzamidine 1, dithiothreitol 1, EDTA 1, EGTA 6, sodium pyrophosphate 15, p-nitrophenyl phosphate 30, and phenylmethylsulfonyl fluoride 0.1 and 0.8 μg/mL leupeptin and 1% Nonidet P-40) for immunoblotting. The cell debris were removed by centrifugation at 12,000g for 10 minutes at 4°C. The samples (20 μg) were treated with 0.5 Laemmli’s sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, and 0.0075% bromophenol blue) followed by heating at 95°C for 3 minutes and then subjected to 8% SDS-PAGE gel for electrophoresis. Protein concentration was measured by protein assay kit from Bio Rad Laboratories AG. The proteins were then transferred onto Immobilon-P filter papers (Millipore AG) with a semidy transfer unit (Hoefer Scientific Instruments). The membranes were then blocked by using 5% skim milk in PBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour and incubated with the antibody against human eNOS (1:600) or with the antibody against human ECE-1 (1:600). The immunoreactive bands were detected by an enhanced chemiluminescence system (Amersham).

#### Endothelial NOS and ECE-1 Protein Expression

Total RNA was isolated by Trizol reagent (Gibco BRL) according to the manufacturer’s instruction. Then 20 μg of total RNA was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred onto a nylon membrane (Hibond-N, Amersham). Blots were hybridized with alkaline phosphatase–labeled cDNA probes, and the signals were detected by chemiluminescent method.

#### ERK Activation

Confluent endothelial cells were rendered quiescent as above for 24 hours and stimulated with thrombin (4 U/mL) from 5 to 30 minutes and then harvested as described above. Activation (phosphorylation) of ERKs was analyzed by Western blots performed as above except that 10% SDS-PAGE and the antibody against phospho-ERK1 and -ERK2 (1:1000) were used.

#### RhoA Membrane Translocation

The confluent and quiescent endothelial cells, as described above, were stimulated with thrombin (4 U/mL) from 5 to 30 minutes. The cells were then washed twice with cold PBS (4°C) and then harvested in PBS buffer (4°C) containing 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.8 μg/mL leupeptin. The cells were then disrupted by brief sonication on ice. The samples were then centrifuged at 500g for 10 minutes at 4°C to remove nucleus. The membrane and cytosol were then separated by centrifugation at 100,000g for 1 hour at 4°C (Beckman Instruments, Inc). The cell membrane was washed once with the buffer described above and then resuspended in buffer containing 100 mmol/L Tris-HCl, 300 mmol/L NaCl, 1% Triton X-100, and 0.1% SDS containing 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.8 μg/mL leupeptin. Equal amounts of protein (10 μg) were loaded into 12% SDS-PAGE gel and electrophoresed. Immunoblotting was then performed as described above except that the antibody against RhoA (1:1000) was used.

#### Endothelium-Dependent and -Independent Relaxations

For functional analysis of eNOS gene expression, aortic rings (5 mm) from WKY rats (9 months old) were isolated and incubated with or without thrombin (4 U/mL) in sterile serum-free DMEM
medium containing 0.2% BSA overnight (16 hours) at 37°C and then suspended in organ chambers in the modified Krebs-Ringer bicarbonate solution (37°C; 95% O2/5% CO2), as previously described (in mmol/L, NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, edetate calcium disodium 0.026, and glucose 11.1 for isometric tension recording with force transducers [Statham Universal UC2]). The aortas were contracted with ET-1, and the endothelium-dependent and -independent responses were examined with acetylcholine and sodium nitroprusside, respectively.

NOS Activity

The cells were harvested with PBS containing 1 mmol/L EDTA and disrupted by sonification. The enzyme reaction (per 100 μg protein) was performed at 37°C in 100 μL of the assay buffer (25 mmol/L Tris-HCl [pH 7.4], 0.6 mmol/L CaCl2, 0.1 μmol/L calmodulin, 2 mmol/L NADPH, 3 μmol/L tetrahydrobiopterin, 1 μmol/L FAD, and 1 μmol/L FMN) containing 0.02 μCi/μL [3H]-arginine (Amersham Pharmacia Biotech) in the presence or absence of 1 mmol/L Nω-nitro-L-arginine methyl ester (L-NAME). After 20 minutes of incubation, the reaction was stopped by the addition of 400 μL of stop buffer (50 mmol/L HEPES [pH 5.5] and 5 mmol/L EDTA). The reaction mixture was applied to Dowex AG50WX-8 column, and [3H]-citrulline of the elute was counted by the scintillation counter. NOS activity was expressed as L-NAME–inhibitable citrulline generation.

ECE Activity

The enzyme reaction (10 μg membrane protein) was carried out at 37°C in 100 μL of assay buffer (20 mmol/L Tris-HCl [pH 7], 0.1% BSA, 20 μmol/L pepstatin A, and 20 μmol/L leupeptin) containing 0.1 μmol/L big ET-1 with or without 100 μmol/L phosphoramidon. After 1 hour of incubation, the reaction was stopped by the addition of 100 μL of 5 mmol/L EDTA. The concentration of ET-1 was determined with an ELISA (Amersham Pharmacia Biotech). ECE activity was expressed as phosphoramidon-inhibitable ET-1 generation.

ET-1 Accumulation

ET-1 accumulation from cells in the medium was measured with an ELISA. ET-1 concentration was normalized to the amount of protein.

Statistics

Data were given as mean±SEM. Relaxations were expressed as percent decrease in tension of the contraction to ET-1. In all experiments, n equals the number of samples. Statistical analysis was performed with unpaired t test in the organ chamber experiment and ANOVA in the other experiments. P<0.05 was considered to indicate a statistical difference.

Results

Effects of Thrombin on eNOS and ECE-1 Expression

Incubation of HUVECs with thrombin (0.01 to 4 U/mL) for 24 hours decreased eNOS protein levels as assessed by immunoblotting but increased ECE-1 levels in a concentration-dependent manner (Figure 1, left). These ef-
Effects of thrombin (4 U/mL) on eNOS and ECE-1 reached maximum at 15 or 24 hours of the incubation, as demonstrated by immunoblotting (Figure 1, right). Incubation with thrombin (4 U/mL) for 24 hours also decreased eNOS mRNA levels and increased ECE-1 mRNA levels, as assessed by Northern blotting (Figure 2). The posttranscriptional regulation of eNOS and ECE-1 mRNA was also determined in the presence of the transcriptional inhibitor actinomycin D (10 μg/mL). Thrombin shortened the half-life of eNOS mRNA, whereas it had no effect on that of ECE-1 mRNA (n=3). *P<0.05 vs control.

PAR1 Activation and eNOS and ECE-1 Expression
Activation of thrombin receptor PAR1 by thrombin receptor–activating peptide (TRAP, SFLLRN) at high concentrations (10 to 100 μmol/L, 24 hours) did not mimic the inhibitory effect of thrombin (4 U/mL) on eNOS expression (Figure 3). In contrast, TRAP increased ECE-1 expression to the similar level as thrombin (4 U/mL) in the endothelial cells (Figure 3).

Rho/ROCK Pathway and Regulation of eNOS and ECE-1 Expression by Thrombin
Furthermore, we analyzed the intracellular signaling mechanisms of thrombin-induced regulation of eNOS and ECE-1 expression in endothelial cells. Thrombin (4 U/mL) stimulated RhoA membrane translocation in a time-dependent manner (Figure 4A). RhoA levels in cytosolic preparation seemed to remain unchanged after thrombin stimulation, possibly because of high level of RhoA in the cytoplasm. Downregulation of eNOS expression by thrombin (4 U/mL, 24 hours) was prevented by Clostridium botulinum C3 exoenzyme (20 μg/mL, Figure 4B), the specific inhibitor of Rho, or by Y-27632 (10 μmol/L, Figure 4C), the specific inhibitor of ROCK, a downstream target of Rho, whereas both inhibitors did not influence upregulation of ECE-1 induced by thrombin (4 U/mL, 24 hours, Figures 4B and 4C).

NOS activity was measured by the l-citrulline synthesis in the cell lysate. Thrombin (4 U/mL) for 24 hours significantly decreased NOS activity (Figure 5). The ROCK inhibitor Y-27632 (10 μmol/L) prevented the decrease in NOS activation induced by prolonged incubation with thrombin (n=3, P<0.05, Figure 5, right); however, it had no effect on NOS activation induced by short incubation with thrombin (Figure 5, left).
Mitogen-Activated Protein Kinase Pathway and Regulation of eNOS and ECE-1 Expression by Thrombin

Thrombin (4 U/mL) activated ERK1 and ERK2 phosphorylation in a time-dependent manner (Figure 6A). Interestingly, the inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK), PD98059 (50 μmol/L), which did not affect thrombin-induced eNOS downregulation, completely prevented the upregulation of ECE-1 by the enzyme (Figure 6B). ECE activity was measured by the conversion of big ET-1 to ET-1 in the membrane fraction. Stimulation with thrombin (4 U/mL for 24 hours) significantly increased ECE activity. MEK inhibitor PD98059 (50 μmol/L) prevented the thrombin-induced ECE activation (n=3, P<0.05) (Figure 7, top). In addition, ET-1 production was evaluated in the conditioned medium with ELISA. ET-1 accumulation was also increased in the medium of the thrombin-treated cells (4 U/mL, 24 hours), and the pretreatment with PD98059 (50 μmol/L) also significantly decreased the thrombin-induced increase in ET-1 accumulation (n=3, P<0.05) (Figure 7, bottom).

Effects of Thrombin on Endothelium-Dependent and -Independent Vasorelaxation in Rat Aortic Rings

In the organ chamber experiment, the level of precontraction induced by endothelin-1 (10^{-8} mol/L) was identical between
the two groups (control, 1.08±0.11 g; thrombin group, 1.15±0.13 g; n=6). The endothelium-dependent NO-mediated relaxation in response to acetylcholine (10^{-7} to 10^{-5} mol/L, n=6) (Figure 8, left) was markedly reduced in rat aorta rings incubated with thrombin (4 U/mL) for 16 hours compared with control rings, which were in parallel incubated in medium without thrombin (P<0.01; maximal relaxation), whereas the endothelium-independent relaxation to the NO donor sodium nitroprusside (10^{-7} to 3×10^{-7} mol/L) was not significantly influenced (n=6, Figure 8, right).

**Involvement of Endogenous NO and ET-1 in the Regulation of eNOS and ECE-1 Expression by Thrombin**

Finally, we examined the impact of endogenous NO and ET-1 on the effects of thrombin using the NOS inhibitor L-NAME and the nonselective ET-1 receptor blocker bosentan. Neither L-NAME (100 μmol/L) nor bosentan (10 μmol/L) had any effects on the downregulation of eNOS and the upregulation of ECE-1 induced by thrombin (see online Figure available in the data supplement at http://www.circresaha.org).

**Discussion**

In the early 1980s, it was recognized that thrombin, the key enzyme in the coagulation cascade, also exhibits profound modulatory effects on vascular tone by activating endothelial cells. Acutely, the enzyme induces marked endothelium-dependent relaxation in arteries from animals and humans.\(^{15,25}\) The mechanism of the endothelium-dependent relaxation in response to thrombin involves activation of eNOS to produce NO from l-arginine or release of prostacyclin from the cyclooxygenase pathway.\(^{8}\) This acute effect of thrombin on the endothelium represents an important feedback mechanism to prevent vasoconstriction and thrombus formation under physiological conditions. Clinical and experimental studies, however, demonstrated that thrombin plays an important role in atherosclerotic process and contributes to thrombus formation and in turn vascular occlusion and myocardial infarction.\(^{26}\)

In clinically manifest human atherosclerosis, eNOS expression is markedly reduced, and this in large part contributes to impaired NOS activity.\(^{3}\) On the other hand ET-1, production is increased under this condition.\(^{8}\) Moreover, ECE-1 expression is also enhanced in atherosclerosis or restenosis after angioplasty.\(^{5}\) The mechanisms or factors that regulate eNOS or ECE-1 expression are not fully elucidated. Taking into the account that thrombin generation occurs in the pathogenesis of atherosclerosis and that the endothelial cells may repeatedly be exposed to high local concentrations of thrombin, particularly in unstable angina and acute myocardial infarction, we hypothesized that prolonged exposure of human endothelial cells in culture to thrombin may affect eNOS and
ECE-1 expression. In this study, we demonstrate for the first time that prolonged exposure of human endothelial cells to thrombin downregulated eNOS and upregulated ECE-1 protein level accompanied by corresponding alterations in the activities of these enzymes. This may represent a novel mechanism of endothelial dysfunction in human atherosclerotic blood vessels.

Thrombin exerts its effects via a family of G protein–coupled protease-activated receptors (PARs). Four such receptors have been identified, three of which (PAR1, PAR3, and PAR4) are activated by thrombin, whereas PAR2 is activated by trypsin. It has been shown that PAR3 and PAR4 mediate the effect of thrombin in mouse platelets, whereas PAR1 and PAR4 mediate activation of human platelets. Whether PAR4 is also expressed in human endothelial cells and which of the receptors is involved in regulating eNOS expression remain to be determined.

Figure 7. Prolonged effects of thrombin on ECE activity and ET-1 accumulation. Thrombin (4 U/mL) treatment for 24 hours significantly increased the conversion from big endothelin-1 to endothelin-1 (top). Thrombin also increased ET-1 accumulation in the medium from cells (bottom). Pretreatment with PD98059 (MEK inhibitor, 50 μmol/L) prevented the thrombin-induced ECE activation and ET-1 accumulation. n=3; *P<0.05 vs control.

Figure 8. Prolonged effects of thrombin on endothelium-dependent relaxation in rat aorta. Endothelium-dependent relaxations in response to acetylcholine (Ach) were markedly reduced in the aortas incubated with thrombin (4 U/mL) for 16 hours, whereas endothelium-independent relaxations induced by sodium nitroprusside (SNP) remained uninfluenced. *P<0.01 vs control (maximal relaxation).
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References

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Thrombin Suppresses Endothelial Nitric Oxide Synthase and Upregulates Endothelin-Converting Enzyme-1 Expression by Distinct Pathways: Role of Rho/ROCK and MAP Kinase

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Figure legend of the online supplement data

Involvement of endogenous NO and ET-1 in the regulation of eNOS and ECE-1 expression by thrombin. In HUVECs, neither L-NAME (NOS inhibitor, 100 µM) nor bosentan (ET-1 receptor blocker, 10 µM) had any effects on the downregulation of eNOS and the upregulation of ECE-1 induced by thrombin (4 U/ml, 24 hours). The top panels shows the representative blots. The middle panel (eNOS) and the bottom panel (ECE-1) show the quantitative analysis with optical densitometry (n=3). * = p < 0.05 vs control.