Regulation of Tissue Factor Expression in Human Microvascular Endothelial Cells by Nitric Oxide

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Background—Tissue factor (TF) is a critical determinant of thrombin generation in normal hemostasis and in atherothrombotic disease. Nitric oxide has both antithrombotic and antiatherosclerotic actions in the vasculature, yet its role in the regulation of TF expression has not been examined.

Methods and Results—To study the effect of endogenous endothelium-derived nitric oxide on TF expression and activity, we induced TF in human microvascular endothelial cells with lipopolysaccharide or interleukin-1β and observed a dose- and time-dependent increase in TF activity and expression by Northern and Western blotting. L-Arginine, the principal substrate for nitric oxide synthases, added to the media suppressed the induction of TF activity significantly (by 66% for lipopolysaccharide induction and by 59% for interleukin-1β induction) at 24 hours. These changes in activity were accompanied by correlative changes in TF protein and steady-state mRNA. D-Arginine had no effect, and inhibition of endogenous nitric oxide production failed to increase TF expression.

Conclusions—These data suggest that enhanced production of endothelium-derived nitric oxide reduces endotoxin- and cytokine-induced expression of TF and, thereby, the prothrombotic phenotype of the endothelial cell. (Circulation. 2000;101:2144-2148.)

Key Words: nitric oxide synthase • proteins • lipids

Tissue factor (TF) is a recognized critical determinant of thrombotic responses in vivo. It is a transmembrane glycoprotein that initiates blood coagulation by binding factor VII with high affinity, thereby promoting factor X activation, thrombin generation, and fibrin formation.1–3 Under normal circumstances, endothelial cells express no or minimal amounts of TF; however, with endothelial cell activation or frank dysfunction, TF expression is significantly enhanced.4–6 Recent studies suggest that the accumulation of TF in atherosclerotic plaque plays a major role in determining plaque thrombogenicity.7–9

Nitric oxide (NO) is produced by normally functioning endothelial cells as a result of the action of the endothelial isoform of NO synthase (eNOS), which oxidizes the amino acid l-arginine to citrulline and NO. Endothelial cells can also be induced to express the inducible isoform of NO synthase (iNOS),10–12 which is a much more catalytically efficient enzyme that is regulated principally at the level of transcription. Regardless of its enzymatic source, endothelial NO has been shown to influence a variety of endothelial responses that are important in normal and pathophysiological vascular function, including the suppression of inflammatory responses, leukocyte adhesion, and platelet-dependent thrombosis.13–15

Both TF and NO synthase expression are upregulated when endothelial cells are activated. Recently, an association between the coagulation and endothelial NO pathways has been reported by Papapetropoulos et al.,16 who showed that factor Xa-induced NO release modulates endothelial cell–dependent vasorelaxation and cytokine gene expression. However, the relation between the TF pathway and NO synthase systems in endothelial cells is, as yet, unknown. Because of the antithrombotic effects of NO, the present study was designed to explore the possible inhibitory role of endothelial NO on endothelial TF expression in response to endotoxin or cytokine exposure.

Methods

Chemicals

Thromboplastin was purchased from Sigma Diagnostics. Interleukin 1β (IL-1β) was purchased from Gibco, Inc. Protein G Plus-Agarose was obtained from Calbiochem. L-Arginine, D-arginine, lipopolysaccharide (LPS), Nω-nitroarginine-l-monoethyl ester (L-NAME), and S2222 were all purchased from Sigma Chemical Co.

Cell Culture

Human microvascular endothelial cells (HMVECs) were obtained from Cell Systems Corp and cultured in CS-C medium supplemented with growth factor and attachment factor (Cell Systems Corp) and with 20% FBS (Gibco). Before experiments, cells were grown to confluence in 24-well microplates or 100-mm tissue culture dishes. All experiments were performed on the second to eighth passage of cells.

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Preparation of Barium Citrate Eluate (Containing Factors VII and X)

Factors VII and X were partially prepared from bovine serum as described by Pitlick and Nemerson.  
Briefly, 7.6% sodium citrate and 15% barium chloride were added to the serum. After 20 minutes of mixing, the mixture was centrifuged at 25 000 g for 20 minutes. The precipitate was washed twice with 5% barium chloride and once with water. The precipitate was then resuspended in 75.5 mL water/L serum. Solid ammonium sulfate (20 g/L serum) was added to the extract. After centrifugation, the same amount of ammonium sulfate was added to the supernatant. The precipitate was collected by centrifugation, resuspended in water (12 mL water/L serum), and dialyzed overnight against 50 mmol/L imidazole HCl, 100 mmol/L sodium chloride, pH 7.0. Aliquots were stored at −80°C. Before use, an aliquot of eluate was dialyzed overnight against the same dialysate as described above.

TF Activity Assay

The surface expression of TF on HMVECs was measured with a 2-stage amidolytic assay as previously described with slight modifications. A 24-well microplate containing confluent HMVECs was washed four times with CS-C medium. CS-C medium (0.20 mL, serum free) was added to the test wells. In the first stage of the assay, 10 μL barium citrate eluate was added to each well and incubated for 10 minutes on a rotating platform (120 cycles/min). In the second stage, 0.1 mL of conditioned medium was combined with 0.05 mL 1 mmol/L S_{2,2′}-1-sulfopropyl mercaptan. The reaction was carried out at 37°C for 30 minutes, after which the OD405 was measured with a Thermomax Microplate Reader (Molecular Devices). TF activity was obtained from a standard curve derived from serial dilutions of rabbit brain thromboplastin assayed in medium. After the assay, the cells were removed from each well with 0.05 trypsin/EDTA and counted with a hemocytometer.

Western Analysis

Monolayers of HMVECs were lysed with immunoprecipitation buffer (1% Triton X-100, 150 mmol/mL NaCl, 10 mmol/mL Tris, pH 7.4, 1 mmol/mL EDTA, 1 mmol/mL EGTA, 0.2 mmol/mL sodium orthovanadate, 0.2 mmol/mL PMSF, and 0.5% NP-40) and sonicated on ice to disperse any large aggregates. Specific antibodies (mouse anti-human TF antibody, Calbiochem, and anti-iNOS and anti-eNOS antibodies, Transduction Laboratories) were added to total lysate. After 1 hour of incubation at 4°C, Protein G Plus-Agarose was added to the lysate to collect the antigen-antibody complexes. Agarose beads were washed twice with immunoprecipitation buffer, resuspended in electrophoresis sample buffer (250 mmol/L Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol), and then boiled for 5 minutes. The beads were pelleted by centrifugation at 16 000 g at 4°C for 3 minutes. The supernatant was loaded onto an SDS-PAGE gel to separate the proteins. Proteins were transferred to nitrocellulose filters and then immunoblotted with specific antibodies. An anti-mouse horseradish peroxidase–conjugated antibody was used as a secondary antibody.

Northen Analysis

Total RNA was isolated from HMVECs with an RNAIsolation System (Promega). RNA was probed with a human TF cDNA kindly provided by Dr Mark Taubman (Mt Sinai School of Medicine, New York, NY); a human eNOS cDNA (Alexis Biochem); or an iNOS cDNA (Alexis Biochem). cDNA probes were [32P]cDPCTP-labeled to 1×10^6 cpm/μg of cDNA (Random Primer Labeling Kit, Stratagene). The blot was prehydrated in a solution containing 50% formamide, 5% Denhardt’s solution, 5% SSPE, 0.1% SDS, and 100 μg/mL salmon sperm DNA at 42°C for 2 hours before addition of the probe; hybridization was performed at 42°C overnight. Human β-actin cDNA-labeled with [32P] was used as a control.

Measurement of Total Nitrite and Nitrate

Total nitrite and nitrate were measured in conditioned medium by the method of Saville with a nitrate reductase step.

Statistical Analysis

Treatment and time course responses were performed by ANOVA techniques. Multiple comparisons were made with either Dunnett’s or Newman-Keuls post hoc tests, where appropriate. Multiple time-course treatment comparisons were performed by 2-way ANOVA. Values given represent mean±SEM.

Results

TF Activity on HMVEC Surface

Confluent HMVECs were treated with LPS (0 to 10 μg/mL) or IL-1β (0 to 100 ng/mL) for 24 hours, after which cell-surface TF activity was measured. As shown in Figure 1, LPS and IL-1β both induced TF activity. The effects of these agents were concentration dependent and occurred maximally after 24 hours of induction. Activity increased approximately 3-fold at the highest concentration of LPS (10 μg/mL), from 34.9±4.2 to 93.4±5.3 mU/10^6 cells, or IL-1β (100 ng/mL), from 33.9±4.3 to 96.2±4.1 mU/10^6 cells.

TF activity increased in a time-dependent fashion, as shown in Figure 2. L-Arginine suppressed these effects by 66% for LPS (from 70.7±2.1 to 50.9±2.4 mU/10^6 cells) and by 59% for IL-1β (from 70.8±1.9 to 53.0±3.6 mU/10^6 cells) and did so maximally by 24 hours of incubation. Neither d-arginine (data not shown) nor N\textsuperscript{N}-nitro-l-arginine methyl ester (L-NAME) had any significant effect on TF activity under these conditions; however, L-NAME coinfused with l-arginine prevented the decrease in TF activity produced by l-arginine (data not shown). l-Arginine supplementation increased NO production by HMVECs (measured as nitrite/nitrate in the conditioned medium) 7.2-fold, from 6.2 μmol/L in control conditions (ie, without l-arginine supplementation) to 44.2 μmol/L (with 1 mmol/L l-arginine supplementation) after 24 hours. Preliminary data also suggest that incubation with an NO donor, 10 μmol/L S-nitrosoglutathione, also suppressed TF activity by 60%.

TF Protein Expression

HMVECs were exposed to various concentrations of LPS (0 to 10 μg/mL) or IL-1β (0 to 100 ng/mL) for 24 hours. Protein extracts from HMVECs were immunoprecipitated with an
anti-human TF antibody to quantify TF protein. Both IL-1β and LPS increased TF protein expression in a concentration-dependent manner, as shown in Figure 3, and did so by 6-fold and 5-fold, respectively, at the maximal concentration of the inducing agent used.

After treatment with l-arginine (1 mmol/L), d-arginine (1 mmol/L), or L-NAME (300 μmol/L) for 45 minutes, HMVECs were exposed to LPS (1 μg/mL) or IL-1β (1 ng/mL), and immunoprecipitation and immunoblotting were repeated. As shown in Figure 4, l-arginine suppressed the expression of TF protein that resulted from activation with LPS or IL-1β, whereas neither d-arginine nor L-NAME had any effect. In addition, the combination of LPS and IL-1β (Figure 4B) further enhanced TF expression, and this enhanced expression was also suppressed by l-arginine.

**TF mRNA Expression**

Using a human TF cDNA probe, we determined the effect of LPS and IL-1β on TF mRNA expression in HMVECs. Steady-state TF mRNA levels increased in response to LPS and IL-1β and did so in a dose-dependent manner. Steady-state TF mRNA levels increased by up to 2-fold, relative to β-actin mRNA, at the highest concentration of the inducing agent (10 μg/mL LPS or 100 ng/mL IL-1β) (Figure 5). These increases in mRNA were suppressed by coincubation with l-arginine, but not d-arginine. L-NAME had no enhancing effect on TF mRNA expression (Figure 6). Importantly, neither l-arginine, d-arginine, nor L-NAME had any effect on TF expression in cells not induced with LPS or IL-1β (Figure 7).

**eNOS/iNOS Protein**

An anti-eNOS antibody and an anti-iNOS antibody were used in immunoblots to measure changes in eNOS and iNOS proteins, respectively, induced by LPS or IL-1β. There was no change in eNOS protein expression after incubation of HMVECs with IL-1β or LPS under these experimental conditions (Figure 8). Similarly, iNOS protein was not induced by LPS or IL-1β used alone under these experimental conditions (data not shown).

**eNOS/iNOS Northern Analysis**

We used a human eNOS cDNA probe and a mouse iNOS cDNA probe to blot RNA of HMVECs after coincubation with LPS and IL-1β. Similar to the immunoblotting experiments, there were no differences in the steady-state levels of eNOS mRNA after LPS or IL-1β treatment compared with
control untreated cells; iNOS mRNA was not induced by pretreatment with either of these agents (data not shown).

Discussion

Endothelial cells modulate the balance between thrombosis and hemostasis.20 This balance, however, can be perturbed by the synthesis and expression of TF. TF expression is associated with life-threatening thrombosis in a variety of diseases, including sepsis, cancer, and atherosclerosis.21 Although quiescent endothelial cells express no or very little TF, TF expression can be significantly induced on the surface of endothelial cells by inflammatory stimuli, such as LPS,18 IL-1β,22 and tumor necrosis factor-α.23 In the present study, we demonstrate that LPS and IL-1β can induce TF activity on the surface of HMVECs. The increase in TF activity in response to these agents was concentration dependent. Moreover, Western and Northern analyses showed that TF protein and mRNA expression, respectively, were increased in HMVECs in a correlative manner.

NO is a cellular signaling molecule that plays a major role in a variety of important biological processes, including the prevention of vascular thrombosis,24 suppression of inflammatory cell-mediated injury,25 and regulation of endothelial integrity and cell proliferation.26,27 NO is synthesized from L-arginine by NO synthase, and cellular NO production is absolutely dependent on the availability of L-arginine.28 The present study shows for the first time that L-arginine, as a substrate for NO synthase(s), suppresses endotoxin- and cytokine-induced expression of TF in HMVECs.

Endothelial cells provide the vascular system with a nonthrombogenic surface.29 Activation of endothelial cells enhances NO production. NO synthesized by endothelial cells not only evokes vasorelaxation but also plays a pivotal role in preventing thrombosis. The antithrombotic actions of NO have, to date, largely focused on its antiplatelet effects,30–33 and deficiencies of endothelium- or platelet-derived NO can lead to a thrombotic diathesis.34,35 Vascular injury also induces the expression of TF at local sites, where it becomes available for plasma factor VII/VIIa binding.36

Dysregulation of NO production had been implicated in the pathogenesis of a number of vascular diseases, including essential hypertension and atherothrombosis. The data in the present study indicate that NO may also reduce thrombotic propensity by interfering with TF expression and, thereby, coagulation. Thus, NO may prevent thrombosis not only by inhibiting platelet function but also by suppressing TF-dependent coagulation.31 Interestingly, the lack of effect of L-NAME in these experiments suggests that basal levels of NO have little effect on the induction of TF by LPS or IL-1β.

We conclude that endothelial NO regulates the expression of TF in endothelial cells. These data suggest that endothelial NO modulates the prothrombotic phenotype of endothelial cells.

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