Thrombin Regulation of mRNA Levels of Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-I in Cultured Human Umbilical Vein Endothelial Cells

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Cultured human umbilical vein endothelial cells release tissue plasminogen activator (t-PA) and type I plasminogen activator inhibitor (PAI-1) in response to alpha thrombin stimulation. In order to study the mechanisms of thrombin stimulation, we measured changes in levels of mRNA for t-PA and PAI-1 following exposure of endothelial cells to 3 U/mL alpha thrombin. Alpha thrombin causes a significant and time-dependent increase in the mRNA levels of both t-PA and PAI-1. Catalytically inactivated diso-

**Phosphophosphate (DIP)-treated thrombin, which binds to high affinity cellular receptors for thrombin yet is catalytically inactive, does not alter t-PA and PAI-1 mRNA levels.** Pretreatment with hirudin, which blocks both the binding and catalytic functions of alpha thrombin, also eliminates the ability of alpha thrombin to increase t-PA and PAI-1 mRNA. As with previously reported data on t-PA and PAI-1 protein release from endothelial cells, both high affinity binding and catalytic activity must be present for thrombin to modulate t-PA and PAI-1 mRNA levels.

**METHODS**

Reagents. Human alpha thrombin (931 U/mg, as determined by the S-2288 assay) (KabiVitrum, Stockholm), and DIP-thrombin (.0777 U/mg) were kind gifts of Dr J. Fenton, NY State Health Department, Albany. The thrombin and DIP-thrombin were diluted in 0.9% pyrogen-free saline (Kendall McGaw, Irvine, CA) containing 1.0 mg/mL fraction V low endotoxin bovine serum albumin (BSA) (Sigma Chemical Co, St Louis) for presentation to the cells. This same solution of BSA in saline, is hereafter called the "control medium." Hirudin treated thrombin was prepared by incubation of 2 units of hirudin (Sigma) per 1 unit of thrombin at 0°C for 20 minutes. The t-PA cDNA probe was a 1.6 kb PstI fragment of plasmid pPA34T-E2, provided by Dr S. Degan, Department of Pediatrics, University of Cincinnati. The PAI-1 cDNA probe was a 2 kb EcoRI fragment of pPAIB6, a gift of Dr D. Ginsburg, Howard Hughes Medical Institute, University of Michigan. The protein S cDNA probe was a 2.2 kb EcoRI fragment of lambda gtl1 clone M117S, provided by Dr E. Cohen of Integrated Genetics, Framingham, MA. The c-sis mRNA was detected with a 1.3 kb fragment containing the v-sis oncogene, provided by Dr D.J. Donoghue, University of California San Diego, La Jolla. For quantitation of the loading of RNA onto the gels, we used an oligonucleotide probe [5'ACGGTATCTGATCGTCTTCGAACC-3'], which hybridizes to 18S rat ribosomal RNA, provided by Dr K.D. Bloch of Massachusetts General Hospital.

**Cell preparation, culture, and characterization.** Endothelial cells were obtained from fresh umbilical cords by collagenase (Worthington Biochemical Corp, Freehold, NJ) digestion using the method of Jaffe et al, and were grown to confluence on 100 mm diameter plastic dishes (Falcon Labware, Becton Dickinson, Oxnard, CA) coated with 1% gelatin (Sigma). Experiments were performed on pooled cells harvested from two to five cords, which had been passaged once or two times using trypsin-EDTA digestion (Biofluids, Rockville, MD). Cells were cultured in M-199 buffered with HEPES (M.A. Bioproducts, Walkersville, MD) with the addition of 20% fetal calf serum (FCS, M.A. Bioproducts), 80 µg/L additional L-Glutamine (Sigma), 50 µg/mL endothelial cell growth...
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Substance (Collaborative Research Inc, Bedford, MA), 100 μg/mL heparin (Sigma), 50 mg/mL gentamicin (GIBCO, Grand Island, NY), 100 U/mL penicillin and 100 μg/mL streptomycin (M.A. Bioproducts). Endothelial cells were characterized by their typical cobblestone morphology and by virtually homogeneous staining with a peroxidase labeled monoclonal antibody to human factor VIII antigen, performed by J. Naftilan of the Department of Pathology, Massachusetts General Hospital.

It is well-documented that small changes in endotoxin concentration can alter production of PAI-1 by cultured endothelial cells. The effect can occur at endotoxin concentrations as low as 10 pg/mL, a level that is unattainable using standard tissue culture reagents. To ensure that endotoxin levels were not a variable in these experiments, we used a Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) to test the serum-free medium for endotoxin content both before and after the addition of thrombin, DIP-thrombin, and the control medium.

Thrombin stimulation. Cells were left at confluence for 48 hours before experimentation. Each experiment was begun by removal of serum-containing medium, washing of the cells twice with M-199 without additives, then addition of serum-free medium. The serum-free medium differed from the serum-containing medium in that 1% FCS (Boehringer Mannheim Biochemical, Indianapolis) was present instead of the FCS, and heparin and endothelial cell growth substance were absent. This formulation of serum-free medium provided superior maintenance of confluent monolayers compared with other formulations. After four hours in serum-free medium, equal volumes of one of the following was added to each culture dish: alpha thrombin at a final concentration of 3 U/mL (3.2 μg/mL), 3 U/mL alpha thrombin that had been pretreated with hirudin, DIP-thrombin at a final concentration of 3.4 μg/mL, or the control medium (1.0 mg/mL BSA in saline). Cells were harvested by trypsin-EDTA digestion at intervals ranging from two to 21 hours.

RNA characterization. Total cellular RNA was extracted by treatment with guanidinium isothiocyanate and centrifugation through cesium chloride. For Northern analysis, 10 μg of RNA per lane was electrophoresed through formaldehyde-agarose gels and transferred to nylon nitrocellulose membranes (Micron Separations, Inc, Westboro, MA). Labeled t-PA, PAI-1, and protein S cDNA probes were prepared by nick translation using [32P]dCTP and were hybridized overnight. These hybridizations and subsequent washes were performed using standard techniques. The 18S oligonucleotide probe was labeled, hybridized, and autoradiographed as previously described except that blots were washed at 37°C instead of 42°C. Blots were autoradiographed using XAR film (Eastman Kodak Co, Rochester, NY) at −80°C. After autoradiography, each of the probes was removed from the blots by incubation in 50% formamide, 1 mol/L EDTA, 0.1 mol/L Tris for ten minutes at 80°C. The autoradiographs were scanned with a 2222-01 Ultrascans XL laser densitometer with an LKB 2400 GelScan software package (LKB Instruments, Inc, Gaithersburg, MD).

ELISA. To determine levels of t-PA and PAI-1 release, cell culture supernatants were collected, centrifuged for 20 minutes at 15,000 g to remove cellular debris, made .01% with Tween 80 (J. T. Baker Co, Phillipsburg, NJ), and frozen at −80°C until assayed. Assays for t-PA and PAI-1 antigen were performed by Dr. D. Collen, Catholic University of Louvain, Belgium, as previously described.

Statistical analysis. In order to estimate the relative abundance of mRNA under control and stimulated conditions, the following calculations were performed: The absorbance reflecting the quantity of hybridization of the cDNA probes to each lane of the Northern blots was divided by the absorbance reflecting the 18S probe hybridization to the same lane, in order to correct for variations in gel loading. Corrected absorbance values were then used to calculate a ratio of thrombin stimulated to control mRNA for each time point.

RESULTS

Northern analysis. Northern blots probed with the t-PA cDNA showed that human alpa thrombin, when present in the cell culture medium at a concentration of 3 U/mL, caused an increase in levels of t-PA mRNA. Message level increased both in comparison with that in unstimulated cells (t = 0) and to t-PA message level in cells that received the control medium (Fig 1). This difference was detectable at about four hours and continued to increase through 12 hours. At 16 and 21 hours, t-PA mRNA levels returned to baseline (t = 0) levels (data not shown). Using the data obtained from this first experiment, we repeated the thrombin stimulation with endothelial cells obtained from four additional groups of umbilical veins. In these experiments, RNA was harvested at...

Fig 1. Alpha thrombin increases t-PA mRNA in cultured human umbilical vein endothelial cells. The t-PA cDNA probe was hybridized to blots with 10 μg per lane of total cellular RNA, harvested at the indicated times. Three units per milliliter of alpha thrombin (A) or the control medium (B) had been added to culture dishes at t = 0. The probe detected a 2.6-kb message. When signal intensity was quantitated by densitometry, corrected for differences in loading, and compared as a ratio to the signal generated by RNA from cells that had been given the control medium, thrombin specific t-PA message level was found to increase through 12 hours (C).
one time point only, six to eight hours after the addition of either the control medium, alpha thrombin, or DIP-thrombin (Fig 2). The addition of alpha thrombin was associated with a mean sixfold increase in t-PA mRNA over this time period ($P < .05$). DIP-thrombin added in equimolar amounts to the alpha thrombin, caused no significant change in t-PA mRNA levels ($P = .83$).

We hybridized the PAI-1 cDNA probe to the same blots (Figs 2 and 3). Again, addition of alpha thrombin resulted in an increase in mRNA levels, in relation both to unstimulated cells ($t = 0$), and to those that received the control medium. Of note, the addition of the control medium was associated with small increases in PAI-1 mRNA, even after correction was made for differences in gel loading. Possible explanations for this increase in PAI-1 mRNA levels include the change from serum-containing to serum-free medium or the presence of trace contaminants in the BSA. Levels of both the 2.4 and 3.4 kb PAI-1 mRNA increased in response to thrombin stimulation, beginning at about two hours and reaching a maximal level at about eight hours. RNA harvested 16 and 21 hours after thrombin stimulation suggested a persistence of these levels compared with $t = 0$ (data not shown). In repeat experiments (Fig 2), a sixfold increase in the 2.4-kb message in response to alpha thrombin stimulation was observed ($P < .005$). The 3.4 kb message increased fourfold ($P < .01$). The difference between the 3.4- and 2.4-kb PAI-1 messages was of borderline statistical significance ($P = .05$). DIP-thrombin stimulated message levels were not significantly different from controls ($P = .75$ and .48, respectively).

To assess whether the observed changes in t-PA and PAI-1 mRNA were due to a nonspecific increase in messenger RNA, blots were hybridized to the protein S cDNA probe (Fig 4B and C). Protein S mRNA levels did not increase with thrombin stimulation, providing evidence against a nonspecific increase in mRNA.

Preincubation of alpha thrombin with hirudin served as an additional control to examine the specificity of the response to thrombin (Fig 5). The ability of thrombin to increase mRNA levels of t-PA and PAI-1 was obliterated by preincubation with hirudin. Incubation of the EC with hirudin alone did not affect the levels of these mRNA species (data not shown).

**ELISA.** To confirm that our protocol of thrombin stimulation resulted in the release of both t-PA and PAI-1 protein, as has been reported by others, we assayed the culture supernatants for t-PA and PAI-1 antigens by ELISA (Fig 6). We demonstrated a significant release of both proteins into the culture medium following the addition of alpha thrombin ($P < .05$ for each).

**Endotoxin assays.** Using the Limulus amoebocyte lysate test, no significant increase in endotoxin levels was
produced by addition of thrombin, DIP-thrombin, or the control medium to the serum-free medium.

**DISCUSSION**

Thrombin has been characterized as the central bioregulatory enzyme in hemostasis, functioning at the fluid, cellular, and vascular levels. In addition to its pivotal role in the cleavage of fibrinogen to form fibrin and in the activation of factors V and VIII, thrombin causes platelet and neutrophil activation, promotes clot stabilization through the activation of factor XIII, and has pleiotropic effects on the endothelial cells of the vascular wall. In addition to hemostasis, thrombin participates in thrombosis through its thrombomodulin dependent activation of the anticoagulant protein C, and potentially through the induction of t-PA release from endothelial cells. The ability of thrombin to be incorporated into fibrin clots, and to be released in active form as the clot is lysed, further extends the potential for thrombin to interact with the endothelium.

Our work further defines a mechanism by which thrombin regulates endothelial cell production of t-PA and PAI-1, two key components of the thrombolytic and hemostatic systems. The data presented here make it clear that thrombin is capable of mediating the balance between coagulation and thrombolysis not only in the extracellular phase, but also through effects on gene transcript levels in the cells of the vascular wall.

Whether the increase in the mRNA levels of t-PA and PAI-1 occurs via stabilization of existing transcripts or through stimulation of the rate of gene transcription is not addressed in these experiments. As mentioned above, nuclear run-off assays have established that thrombin induces c-sis gene transcription in endothelial cells. Further experiments are required in order to examine whether thrombin also increases transcription rate of the t-PA and PAI-1 genes.

The data presented here also expand our understanding of the regulation of the t-PA and PAI-1 genes. Agents that have been shown to increase levels of specific t-PA and PAI-1 mRNA include phorbol myristate acetate in HeLa cells, Bowes melanoma cells, follicle stimulating hormone and luteinizing hormone in rat granulosa cells, and dexamethasone in human mammary carcinoma cells. Elevations in PAI-1 mRNA levels in human endothelial cells have been produced by interleukin 1, tumor necrosis factor, and...
LPS. Dexamethasone treatment of human fibrosarcoma cells increases t-PA and PAI-1 mRNA through the stimulation of transcription of both genes. Since these agents (steroid and polypeptide hormones, phorbol esters, and LPS) appear to act through different intracellular pathways, a unified model of the regulation of t-PA and PAI-1 transcripts is not yet apparent. Genomic clones of both t-PA and PAI-1 have now been reported, allowing more direct studies of transcriptional regulation. In addition, discovery of large (521 bp) highly homologous (81%) 5' flanking regions in the t-PA and PAI-1 genes is consistent with the hypothesis that these genes can be coordinately regulated. As more becomes known about possible cis-acting sequences and trans-acting factors involved in the regulation of these genes, a more complete view of the regulation of t-PA and PAI-1 transcripts should be attainable.

Our data also demonstrate that an active catalytic site as well as receptor binding are required for thrombin to effect an increase in t-PA and PAI-1 mRNA levels. Hirudin, which blocks both catalytic activity and binding of thrombin to the high-affinity receptor, and DIP inactivation of the thrombin catalytic site both blocked the thrombin associated mRNA increases. This is consistent with earlier data on thrombin stimulation of t-PA and PAI-1 protein release. DIP or hirudin treatment of thrombin eliminates its ability to increase t-PA release from endothelial cells. Gamma thrombin, which has an active catalytic site but cannot bind to high-affinity receptors, is <5% as potent as alpha thrombin in its ability to stimulate t-PA release. Similarly, hirudin treated thrombin and gamma thrombin do not stimulate PAI-1 release from endothelial cells.

Our findings that both the ability to bind and catalytic activity must be intact for thrombin to mediate increases in transcript levels is consistent with the "two signal" hypothesis that was generated to explain thrombin regulation of mitogenesis in fibroblasts. Both functions are required for the thrombin stimulation of mitogenesis, and binding and catalytic activity have each been correlated with a separate set of intracellular signals. High-affinity binding causes increased intracellular cAMP, and the presence of catalytic activity results in increased phosphoinositide turnover and calcium mobilization. These signals can be given by either one molecule with both high-affinity binding and catalytic activity or by two different molecules, each supplying only one of the functions. Our data suggest that the "two signal" hypothesis may apply to the regulation of mRNA levels in endothelial cells as well. It will be of interest to examine the nature of the signals produced by thrombin in endothelial cells, and to determine if the effects of alpha thrombin on mRNA levels can be duplicated by a combination of catalytically active and high-affinity binding thrombin derivatives. Contrary to this "two signal" model, Daniel et al found that DIP-thrombin was equivalent to alpha thrombin in its ability to increase c-sis mRNA levels in renal microvascular endothelial cells. When we probed our blots with a c-sis cDNA probe we found that treatment with alpha thrombin but not DIP-thrombin resulted in increased c-sis mRNA levels. Whether this difference in the ability of DIP-thrombin to increase mRNA levels is due to a variability between cell types, specific experimental conditions, or residual proteolytic activity in the thrombin used by Daniel et al is not apparent.

In summary, we have extended the potential role of thrombin in the regulation of coagulation by showing that it is capable of acting on the vascular endothelium to increase levels of mRNA for t-PA and PAI-1. This action requires catalytic activity and is specific, in that thrombin does not increase the mRNA for protein S. It will be interesting to explore the effects of thrombin on the transcript levels of other endothelial derived procoagulant and anticoagulant proteins, as well as the pathways by which this regulation occurs.

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REFERENCES


50. Gordon EA, Fenton JW, Carney DH: Thrombin-receptor occupancy initiates a transient increase in cAMP levels in mitogenically responsive hamster (NIL) fibroblasts, Ann NY Acad Sci 485:249, 1984


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