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

By David Dichek and Thomas Quertermous

Cultured human umbilical vein endothelial cells release tissue plasminogen activator (t-PA) and type 1 plasminogen activator inhibitor (PAI-I) 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-I 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-I. Catalytically inactivated diso-

Thrombin Has Extensive Actions on Vascular Endothelial Cells. It increases neutrophil adherence to endothelial monolayers and increases the permeability of these monolayers to macromolecules. Exposure to thrombin increases endothelial cell release of platelet activating factor, prostacyclin, factor VIII antigen, interleukin-1, thromboplastin, tissue-type plasminogen activator (t-PA), and the rapid plasminogen activator inhibitor type 1 (PAI-1). The thrombin induced release of t-PA and PAI-I by cultured human umbilical vein endothelial cells is dependent on both RNA and protein synthesis, suggesting that thrombin may act to increase levels of messenger RNA (mRNA); however, this has never been directly shown.

A specific increase in levels of mRNA encoding c-sis and the A chain of platelet derived growth factor in response to thrombin stimulation has recently been shown by nuclear run-off studies to be due to increased transcription rather than stabilization of existing message. An increase in specific mRNA for a potent vasoactive peptide, endothelin, has also been associated with thrombin stimulation. These studies support the hypothesis that many of the previously reported actions of thrombin on cultured endothelial cells (EC) are mediated at the transcriptional level. Thrombin activates the intracellular second messengers inositol triphosphate and diacylglycerol, which have been shown in other settings to influence gene transcription.

We examined the effect of thrombin stimulation of cultured human umbilical vein endothelial cells on levels of mRNA for t-PA and PAI-I. We found a time-dependent increase in both of these mRNA species following addition of 3U/mL alpha thrombin to the culture medium. Disosfluoro-

phosphate (DIP)-treated thrombin, which binds to high affinity cellular receptors for thrombin yet is catalytically inactive, does not alter t-PA and PAI-I 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-I mRNA. As with previously reported data on t-PA and PAI-I protein release from endothelial cells, both high affinity binding and catalytic activity must be present for thrombin to modulate t-PA and PAI-I mRNA levels.

METHODS

Reagents. Human alpha thrombin (931 U/mg, as determined by the S-2288 assay) (KabiVitrum, Stockholm), and DIP-thrombin (0.077 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 pPA34'T provided by Dr S. Degan, Department of Pediatrics, University of Cincinnati. The PAI-I cDNA probe was a 2 kb EcoR1 fragment of pPAI6, 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 gt11 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 rRNA 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 one 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

From the Cardiac Unit, Department of Medicine, Massachusetts General Hospital, Boston.

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Address reprint requests to Thomas Quertermous, MD, Cardiac Research, Jackson 13, Massachusetts General Hospital, Fruit St, Boston, MA 02114.

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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.26 This effect can occur at endotoxin concentrations as low as 10 pg/mL,27 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 (Asso-

Massachusetts General Hospital.

To test the serum-free medium for endotoxin content, we used a Limulus amoebocyte lysate assay (Asso-

Heparin (Sigma), 50 mg/mL gentamicin (GIBCO, Grand Island, NY), 100 U/mL penicillin and 100 μg/mL streptomycin (M.A. Bioproducts). To ensure that endotoxin was not a variable in these experiments, we used a Limulus amoebocyte lysate assay (Asso-

RESULTS

Northern analysis. Northern blots probed with the t-PA cDNA showed that human alpha 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

RNA characterization. Total cellular RNA was extracted by treatment with guanidinium isothiocyanate and centrifugation through cesium chloride.29 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.29 The 18S oligonucleo-

tide probe was labeled, hybridized, and autoradiographed as previously described29 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 2222-011 Ultrascan 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 0.1% with Tween 80 (J. T. Baker Co, Phillipsburg, NJ), and frozen at −80°C until assayed. Assays for t-PA and PAI-1 antigens were performed by Dr D. Collen, Catholic University of Louvain, Belgium, as previously described30,31

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,$^8,9$ 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
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 thrombolysis 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.

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...
cells increases t-PA and PAI-1 mRNA through the stimulation of transcriptional regulation. In addition, the discovery of large numbers of cis-acting sequences 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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