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-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 disosfluorophosphate (DIP)-treated thrombin, which binds to high affinity cellular receptors for thrombin yet is catalytically inactive,

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-1 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 been reported for human microvascular endothelial cells. The increase in c-sis message has been reported for human microvascular endothelial cells on levels of t-PA and PAI-1. We found a time-dependent increase in mRNA levels. In addition, an active thrombin catalytic site is required for these increases in mRNA to occur.

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 pPA34'F2 provided by Dr S. Degan, Department of Pediatrics, University of Cincinnati. The PAI-1 cDNA probe was a 2 kb EcoRI fragment of pPA1B6, 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 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 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...
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 (MA. 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. This 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.

There are different methods to analyze the effect of thrombin on endothelial cells. One method is to use 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 different times and analyzed by Northern blotting.

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 different times and analyzed by Northern blotting.

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. Due to the large variance in the absorbance data, log transformations of the data were performed and paired t tests were performed on the transformed values. The t-PA and PAI-1 antigen levels in the supernatant of the control and thrombin treated cells were compared by a paired t test. Analysis was performed using an RS/1 statistical package (Bolt, Beranek, and Newman, Inc, Cambridge, MA).

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).
Fig 2. Mean increases of mRNA for t-PA and PAI-1, following addition of alpha thrombin or DIP-thrombin to cultured human umbilical vein endothelial cells. Relative mRNA levels were calculated from a total of five experiments for alpha thrombin vs control medium, and three experiments for DIP-thrombin vs control medium. mRNA levels under control conditions were arbitrarily assigned a value of 1, and the other levels were calculated as multiples of these values. In all experiments correction for loading was performed using the results of the 18S rRNA probe hybridization.

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
CONTROL

A

B

2 4 8

f-PA

4 8

f2hr

ci.-THROMBIN

2 4 8

f2hr

CONTROL

2 4 8

f2hr

Protein S

Protein S

Fig 4. Hybridizations of (A) the 18S rRNA oligonucleotide probe and (B) the protein S cDNA probe to the same blot as shown in Figs 1 and 3. (A) The variations in loading that were revealed by the 18S probe hybridizations were used to correct signal intensities measured from the other hybridizations to this blot. (B) The protein S probe detected a 3.5 kb message. As seen in B and C, alpha thrombin does not increase protein S mRNA in cultured human umbilical vein endothelial cells.

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-I have now been reported, allowing more direct studies of transcriptional regulation. In addition, the 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.

ACKNOWLEDGMENT

We are grateful to Kenneth Bloch, Edgar Haber, and Jeffrey Michel for helpful discussions. We appreciate the gifts of cDNA probes from S. Degan, M. Ginsburg, and E. Cohen, and of alpha thrombin and DIP-thrombin from J. Fenton. We thank the obstetrics staff at The Cambridge Hospital for providing the umbilical cords, and D. Colleen for performing the ELISA.

REFERENCES

THROMBIN REGULATES ENDOTHELIAL CELL mRNA


Thrombin regulation of mRNA levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in cultured human umbilical vein endothelial cells

D Dichek and T Quertermous

Updated information and services can be found at:
http://www.bloodjournal.org/content/74/1/222.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml