Thrombin Induction of Plasminogen Activator–Inhibitor in Cultured Human Endothelial Cells

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Abstract

We have examined the effect of thrombin on the activity of plasminogen activator (PA) and plasminogen activator–inhibitor (PA-I) in medium conditioned by primary cultures of human umbilical vein endothelial cells. PA activity was measured by fibrinolytic and esterolytic assays, and total tissue-type PA (tPA) antigen by radioimmunoassay. Net PA-I activity was assayed by titration of human urokinase esterolytic activity. Incubation of confluent endothelial cell cultures with thrombin for 24 h caused a sixfold increase in PA-I activity. The effect of thrombin was half-maximal at ~0.4 U/ml (<4 nM), and required concomitant RNA and protein synthesis. The stimulation of PA-I activity required active α-thrombin and was not obtained with γ-thrombin nor with thrombin catalytically inactivated with hirudin. Because of the excess of PA-I, PA activity was not measurable in either control or thrombin-treated cells. Thrombin did, however, increase medium concentration of tPA antigen by approximately fourfold. The thrombin-induced PA-I inhibited both tPA and urokinase, did not lose activity upon acidification, and was stable to sodium dodecyl sulfate and thiol reduction. We conclude that physiologic concentrations of thrombin increase both PA-I activity and tPA antigen in medium conditioned by human umbilical vein endothelial cells. Because there was always a several-fold increase in the net activity of free PA-I, these observations suggest that the net effect of thrombin is to decrease fibrinolytic activity in human endothelial cells. Thus, thrombin, in addition to its role in coagulation, may protect clots from premature lysis by increasing the amount of a specific fibrinolytic inhibitor.

Introduction

The vascular endothelium, in addition to its well-recognized role in hemostasis, plays an important role in fibrinolysis (1). Human and bovine endothelial cells secrete both plasminogen activators (PAs)[1] and a specific rapidly acting inhibitor of plasminogen activator (2–5). Thrombin, a key component of the coagulation system (6), can also modulate fibrinolysis. Thrombin directly causes the release of plasminogen activator–inhibitor (PA-I) from human platelets (7) and decreases PA activity in cultured bovine aortic endothelial cells (8). The recently reported activation of protein C by thrombin (9) suggests that thrombin may paradoxically enhance fibrinolysis. The objective of this study was to examine the thrombin regulation of PA and PA-I activity in cultured human endothelial cells.

We have reported previously that glucocorticoids rapidly decrease PA activity in cultured rat hepatoma cells secondary to a 10-fold induction in the amount of PA-I without any decrease in the amount of PA (10–13). We report here that physiologic concentrations of thrombin cause a time- and concentration-dependent increase in the activity of PA-I in serum-free medium conditioned by primary cultures of human umbilical vein endothelial cells. Although thrombin also causes an increase in the release of tissue-type PA (tPA) antigen, there is always a several-fold increase in the net activity of free PA-I so that the net effect of thrombin may be to decrease fibrinolytic activity. Thus thrombin, in addition to its role in coagulation, may protect clots from premature lysis by increasing the amount of a specific fibrinolytic inhibitor.

Methods

Materials. Two preparations of human α-thrombin were used in these studies. One (2,666 NIH U/mg) was purchased from Sigma Chemical Co., St. Louis, MO; the other (3,767 U/mg) was kindly provided by Dr. J. W. Fenton II, New York Department of Health, Albany, NY, who also provided γ-thrombin (0.92 U/mg). Recombinant human tPA was a generous gift from Genentech, Inc., South San Francisco, CA, and rabbit anti-human melanoma tPA antiserum was kindly provided by Dr. Desire Collen, University of Leuven, Belgium. Plasminogen was prepared from oudated human plasma by affinity chromatography on lysine-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) by the method of Deutsch and Mertz (14). High molecular weight human urokinase, bovine fibrinogen, Cbz-L-lysyl thiobenzyl ester, and 5,5′-dithiobis (2-nitrobenzoic acid) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Actinomycin D was obtained from Merck and Co., Rahway, NJ, and hirudin and cycloheximide were purchased from Sigma Chemical Co. Para-aminobenzamidine–Sepharose CL 4B and Iodgen were purchased from Pierce Chemical Co., Rockford, IL. Protein A was purchased from Miles Laboratories, Naperville, IL; Na ^{125}I from New England Nuclear, Boston, MA; and ^{125}I-labeled human fibrinogen (140 μCi/mg) from Amersham Corp., Arlington Heights, IL.

Cell culture. Endothelial cell cultures were obtained from Dr. Stephen Weiss' laboratory at the University of Michigan. Endothelial cells were prepared from human umbilical cord veins by collagenase treatment as described by Jaffe et al. (15). All preparations were made from cells combined from several umbilical cords. Approximately 10^4 cells were plated on gelatin-coated 35-mm dishes in 2 ml of M199 (Gibco, Grand Island, NY) containing Hanks salts, 25 mM Hepes, penicillin, streptomycin, and fungizone. The medium was supplemented with 100 μg/ml endothelial cell growth supplement (16) purchased from Collaborative Research, Waltham, MA, and 20% human serum. Human serum was collected from a panel of donors and was not heat-inactivated. The cul-

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1. *Abbreviations used in this paper:* PA, plasminogen activator; PA-I, PA-inhibitor; tPA, tissue type PA: uPA, urokinase-like PA.

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tures were allowed to grow to confluence (5–7 d) at which time they contained ~8 × 10^5 cells/dish (100–150 μg protein/dish). Confluent primary cultures were used for all experiments. The cultures were washed three times with 2 ml serum-free medium at 37° and incubated in 2 ml serum-free medium with additions as described in the legends to the figures and tables. After incubation, medium was collected, made 0.01% with Tween 80, centrifuged at 1,200 *g* to remove cell debris, and frozen at −20° until assayed. Before assay, thrombin activity was neutralized by incubation with hirudin (2 U/unit thrombin) for 20 min at 0°.

Assay of PA activity. Primary cultures of human endothelial cells contain and release tPA but not urokinase-like PA (uPA) (2–4). PA activity was measured either by an ^{125}I-fibrin plate assay as described previously (13) or by a modification of the two-step esterolytic assay described by Coleman and Green (17). The latter assay was adapted for measurement of tPA activity by the addition of 220 μg/ml of cyanogen bromide–digested fibrinogen (18) and was sensitive to as little as 5 pg of tPA.

Radioimmunoassay of tPA. Recombinant human tPA was iodinated to a specific activity of ~36 μCi/μg by the Iodogen method (19). Benzamidine was present during iodination to protect the active site and iodinated tPA was purified by affinity chromatography on *p*-aminobenzamidine-Sepharose 4B with elution of active enzyme by 1 mM HCl, pH 3, with 0.1% bovine serum albumin. Anti–human melanoma tPA IgG was prepared from rabbit antiserum by chromatography on protein A-agarose (20). Radioimmunoassay was carried out by modification of the method of McGregor and Prowse (21). tPA (10–1,000 pg in 120 μl) was incubated overnight at 4°C with 15 μl of specific IgG (1.6 ng). 15 μl of ^{125}I-labeled tPA (~20,000 cpm) was then added and incubated for 1 h at 37°C. One ml of 0.25% protein A was added and the incubation continued for 15 min at room temperature before centrifuging bound material at 12,000 *g* for 5 min. Standard curves were analyzed by logit transformation in which logit B was plotted versus log tPA concentration. Logit B = ln(B/100-B), where B is the radioactivity bound in a test sample expressed as a percentage of the radioactivity bound in the zero standard sample, corrected for nonspecific binding. Standard curves were linear between 10 and 1,000 pg, with 50% displacement at ~120 pg. Nonspecific binding was <2%. This assay measures both free tPA and tPA bound to PA-I and can detect as little as 25 pg of tPA.

Assay of PA-I activity. Inhibitory activity was measured by titration of residual uPA activity using the coupled esterolytic assay as described previously (11, 17). Serial dilutions of conditioned medium made with unconditioned serum-free medium containing hirudin were incubated with uPA for 10 min at 37°C before initiation of the activation reaction by addition of plasminogen. Each assay was performed in duplicate. That dilution that inhibited 50% of the activity of 0.54 fmol (2 milliPloug units [mPU]) of uPA was defined as having 0.27 fmol (1 mPU) of PA-I activity. Results are expressed as fmol/cm^2 surface area.

Results

PA activity was not measurable in medium conditioned by either control or thrombin-treated cells, as assayed by both the ^{125}I-fibrin plate assay or the coupled esterolytic assay. Presumably this reflects the fact that tPA released from human endothelial cells is bound to PA-I, which is present in excess in medium conditioned by these cells. In other experiments (not shown) endothelial cells were plated directly on dishes coated with ^{125}I-fibrin and grown to confluence in plasminogen-depleted serum. The cultures were then incubated in plasminogen-containing serum in the presence or absence of thrombin. In neither case was any fibrinolytic activity detected above background release. In contrast, PA-I activity was readily measurable and was increased markedly by incubation of endothelial cells with thrombin.

Fig. 1 shows the time-course of thrombin stimulation of PA-I activity in medium conditioned by human endothelial cells. During the first 3 h of incubation no increase over control levels

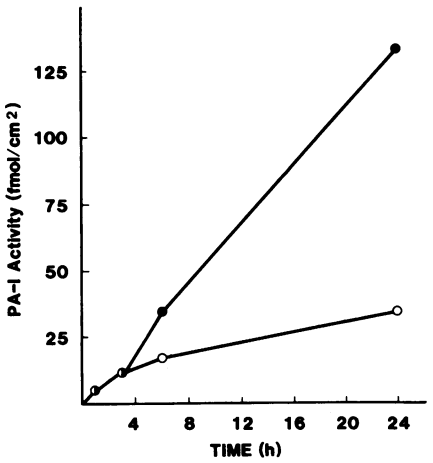

Figure 1. Time course of thrombin stimulation of PA-I activity. Confluent cultures of endothelial cells were incubated without (open circles) or with (closed circles) 2 U/ml thrombin for the times indicated. Medium was made 0.01% with Tween 80, centrifuged to remove cell debris, and frozen. PA-I activity was measured by titration of residual uPA activity as described in Methods. Each point represents the average of duplicate assays performed on duplicate dishes.

of inhibitor activity was observed; however, at 6 h, the medium from thrombin-treated cells contained approximately twice the levels found in control, and after 24 h incubation, PA-I activity was increased several-fold over that seen in the medium conditioned by control cells. After 24 h incubation with thrombin, PA-I activity was 5.8±1.1-fold greater than that in control cells (mean±SE, n = 10).

The concentration dependence of the thrombin stimulation of PA-I accumulation is shown in Fig. 2, which represents the mean±standard error of four separate experiments. The effect of thrombin on PA-I activity was detectable at concentrations

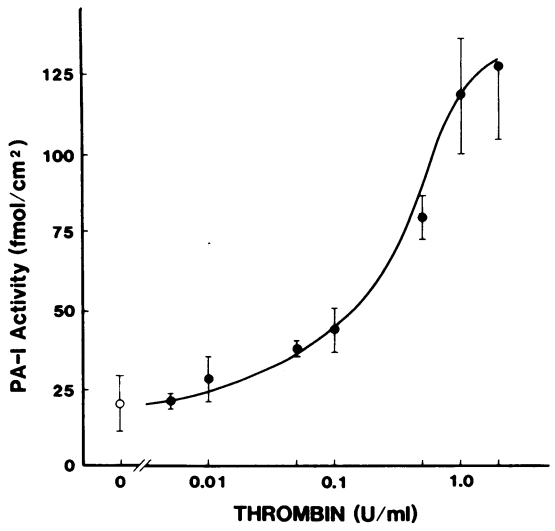

Figure 2. Concentration dependence of the thrombin stimulation of PA-I activity. Confluent cultures of endothelial cells were incubated for 24 h without (open circles) or with (closed circles) the indicated concentrations of thrombin. PA-I activity in the medium was measured as described in Methods. Each point represents the mean±SE from four separate experiments.

Table I. Requirement for Catalytically Active α-Thrombin for the Stimulation of PA-I

Treatment	PA-I activity
	fmol/cm²
Control	18.8±0.9
α-Thrombin	53.3±4.0
γ-Thrombin	19.0±2.3
Hirudin-thrombin	19.8±2.1

Endothelial cell cultures were incubated for 24 h without additions (control), or with 1 U/ml α-thrombin, or with γ-thrombin at an equivalent protein concentration (265 ng/ml), or with 1 U/ml α-thrombin that had been catalytically inactivated by prior incubation for 20 min at 0°C with 2 U/ml hirudin. PA-I activity in the medium was assayed as described in Methods. Inhibitor activity is presented as the mean±SE from four separate experiments.

above 0.01 NIH U/ml and was maximal at 1-2 U/ml. The effect was half-maximal at ~0.4 U/ml (<4 nM). The concentration of active thrombin in the circulation during clotting is not known with certainty, but is estimated to be 60–90 nM (6).

As shown in Table I, the stimulation of PA-I activity requires catalytically active α-thrombin. γ-Thrombin, which hydrolyzes nitroanalide substrates with an efficiency close to that of α-thrombin, but is >2,000-fold less effective in clotting fibrinogen (6, 22) and in releasing fibrinopeptide A (Shafer, J. A., personal communication), did not stimulate PA-I activity. Thrombin catalytically inactivated with hirudin also failed to enhance PA-I activity, indicating the requirement for catalytically active α-thrombin.

The stimulation of PA-I activity by thrombin requires concomitant protein and RNA synthesis, as indicated by the fact that it is blocked by cycloheximide and actinomycin D (Table II). 50 μM cycloheximide, which inhibits amino acid incorporation into protein by more than 75%, completely blocked the accumulation of PA-I activity in medium conditioned by both control and thrombin-treated endothelial cells. Actinomycin D at 0.16 μM, which inhibited DNA-dependent RNA synthesis by 90%, similarly blocked the appearance of PA-I activity in the medium.

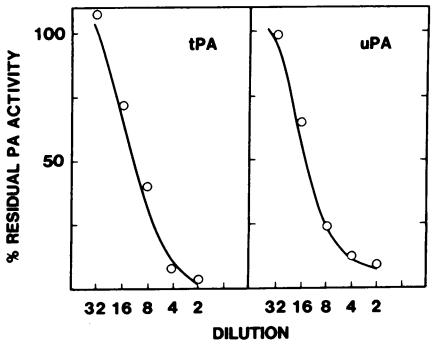

Figure 3. Inhibition of both tPA and uPA by thrombin-induced PA-I. Medium pooled from two endothelial cell cultures incubated with 1 U/ml thrombin for 24 h was assayed for inhibitor activity against both tPA and uPA using the coupled esterolytic assay. Inhibition of uPA was assayed as described in Methods; 100% activity represents that of 0.54 fmol uPA. Inhibition of tPA activity was determined using the same assay in the presence of 220 μg/ml cyanogen bromide–digested fibrinogen; 100% activity represents that of 0.5 fmol tPA. Each point represents the average of duplicate assays.

The properties of the thrombin-induced endothelial cell PA-I are the same as those previously described by us for the PA-I induced by glucocorticoids in rat hepatoma cells (10–13) and by others for the PA-I in human plasma (23–26), platelets (7), and cultured human (2–4) and bovine endothelial cells (5, 27). As shown in Fig. 3, the thrombin-induced inhibitor rapidly inhibits both tPA and uPA with similar potency. PA-I activity in this sample was 63 fmol/cm² when assayed against tPA, and 73 fmol/cm² against uPA. Second, as shown in Table III, the inhibitor did not lose activity upon incubation at pH 3 for 1 h at room temperature, a treatment which destroys the activity of most plasma protease inhibitors, including α_2 anti-plasmin (28), as well as the activity of the placenta PA-I (29) and of a fibroblast PA-I called protease nexin (30), but not that of either the endothelial (2–5) or hepatoma (10–12) PA-I. Finally, as shown in Fig. 4, the inhibitory activity was also stable to sodium dodecyl sulfate (SDS) and thiol reduction. Incubation of medium from thrombin-treated cultures with 0.1% SDS and 0.1% 2-mercaptoethanol for 1 h at 37°C, followed by extensive dialysis, in fact, increased inhibitory activity approximately eightfold (from 112

Table II. Effect of Cycloheximide and Actinomycin D on the Thrombin Induction of PA-I Activity

Inhibitor	PA-I activity	
	Control	Thrombin
	fmol/cm²	*fmol/cm²*
None	22±7	95±18
Cycloheximide	<6	<6
Actinomycin D	6±1	<6

Endothelial cell cultures were incubated for 30 min with 0.1 mM cycloheximide, 0.16 μM actinomycin D, or no inhibitor, and then for a further 18–24 h with or without 2 U/ml thrombin. PA-I activity was assayed in the medium as described in Methods, and is presented as the mean±SE from three separate experiments. The limit of detection for this assay was 6 fmol/cm².

Table III. Acid Stability of the PA-I

	PA-I activity	
	Control	Acidified
	fmol/cm²	*fmol/cm²*
Control	25	20
α-Thrombin	123	112

To 1 ml of medium from control or thrombin-treated endothelial cells, 30 μl of 1 N HCl was added to reduce the pH to 3.2. After 1 h incubation at room temperature, 25 μl of 1 N NaOH was added to restore the pH to 7.4. As a control, 1 ml of medium from thrombin-treated and untreated cells was incubated for 1 h with a mixture of HCl and NaOH. PA-I activity was assayed by titration of uPA esterolytic activity as described in Methods. Each datum represents the mean of duplicate assays on duplicate samples.

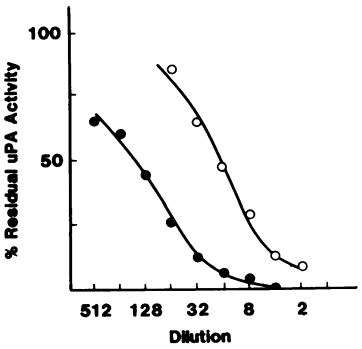

Figure 4. Activation of thrombin-induced PA-I by SDS and 2-mercaptoethanol. Medium from a thrombin-treated endothelial cell culture was adjusted to pH 5.6 with HCl and incubated for 1 h at 37°C without (open circles) or with (closed circles) 0.1% SDS and 0.1% 2-mercaptoethanol. Both samples were then dialyzed for 20 h against phosphate buffered saline, pH 7.4, containing 0.01% Tween 80 and 0.02% sodium azide. PA-I activity was assayed by titration of residual uPA activity as described in Methods.

to 896 fmol/cm^2 in this sample). A similar activation of PA-I has been observed in both rat hepatoma cells and bovine endothelial cells (31).

Although no PA activity could be measured in the medium conditioned by either control or thrombin-treated cells, Levin and co-workers (32) have previously reported that thrombin increases the concentration of tPA antigen in medium conditioned by thrombin-treated cells. Primary cultures of human endothelial cells produce only tPA but not uPA (2–4). We measured tPA antigen by a radioimmunoassay that detects and quantitates both free tPA as well as tPA bound to PA-I. To demonstrate this, 2 ng tPA was mixed with a 500 μl sample of endothelial cell–conditioned medium containing sufficient PA-I activity (~200 fmol) to completely neutralize the added tPA, and the mixture was incubated for 1 h at 37°C to allow complex formation. No tPA activity was measurable in the endothelial cell medium before or after addition of the exogenous tPA. Radioimmunoassay of tPA antigen revealed 0.27 ng tPA in the medium before, and 2.15±0.23 ng (mean±SE of four dilutions assayed in duplicate) after adding 2 ng tPA to the medium. Using this assay, we have confirmed that thrombin increases tPA concentration in medium conditioned by human endothelial cells by approximately fourfold (from 2.6±0.6 to 9.2±1.8 fmol/cm^2; mean±SE, n = 6). The time course and concentration dependence of the thrombin enhancement of tPA antigen levels in the medium (data not shown) is similar to that for PA-I activity. It should be noted, however, that the concentration of tPA in the medium from both control and thrombin-treated cells is approximately 10-fold lower than the concentration of active PA-I.

Discussion

We report here that physiological concentrations of thrombin increase PA-I activity, and tPA antigen, in serum-free medium conditioned by human umbilical vein endothelial cells. Because there was always a several-fold increase in the net activity of free PA-I, the net effect of thrombin may be to decrease fibrinolytic activity in medium conditioned by human endothelial cells. Thus, thrombin, in addition to its role in coagulation, may protect clots from premature lysis by increasing the amount of a specific fibrinolytic inhibitor.

These observations provide further evidence that human vascular endothelium may play a significant role in fibrinolysis as well as in hemostasis, and that thrombin can modulate the fibrinolytic system in addition to its multiple effects on hemostatic processes. Thrombin is known to cause release of PA-I from human platelets (7) and to inhibit PA activity in medium conditioned by bovine aortic endothelial cells (8). Our observations that thrombin can decrease fibrinolytic activity in human endothelial cells by inducing PA-I are consistent with the conclusion that thrombin both enhances hemostasis and inhibits fibrinolysis.

The effects of thrombin on fibrinolysis, however, may be more complex. Thrombin, in the presence of thrombomodulin, activates protein-C, which enhances fibrinolytic activity in several experimental systems (9). Incubation of cultured human endothelial cells with active protein-C decreases the accumulation of PA-I in the medium, which secondarily increases PA activity; there is no effect on the accumulation of tPA antigen. The effect of active protein-C can be demonstrated in cell-free medium and may result from the direct proteolytic cleavage of PA-I (33). The thrombin stimulation of tPA antigen release by human endothelial cells reported by Levin et al. (32), and by us, also suggests that thrombin might have fibrinolytic effects as well.

It is becoming apparent that fibrinolysis is regulated, at least in part, by a balance between PA and PA-I. The 10-fold excess of inhibitor relative to PA observed in cultured human umbilical vein endothelial cells by us and others (2–4) is not an exact representation of the in vivo situation; however, there is substantial evidence that in vivo, most if not all of the circulating tPA is bound to PA-I (34). Estimates of blood tPA activity (23, 24, 34–36) range from 0.2 to 2 ng/ml (3–30 pM); whereas tPA antigen (21, 34) is ~5–10 ng/ml (75–150 pM). Measurement of PA-I activity has varied widely in different individuals and has ranged from 0 to 120 ng/ml (0–2.4 nM) with an average of ~1 nM (23–26, 34, 35). Thus, tPA in the circulation also exists predominantly as a 100,000-D complex with inhibitor.

Other factors, in addition to thrombin, can regulate PA-I activity in blood. Endotoxin enhances PA-I release by endothelial cells in vivo and in vitro (37), and the anabolic steroid stanozolol has been shown to increase fibrinolytic activity in vivo by decreasing PA-I activity rather than by increasing the amount of tPA (38). Finally, there is increasing evidence that PA-I plays an important role in thromboembolic disease (26, 39), and a family has been reported with an autosomal dominant thromboembolic disorder presumably secondary to increased levels of circulating PA-I (40).

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