Induction of Vascular Smooth Muscle Cell Expression of Plasminogen Activator Inhibitor-1 by Thrombin

Hiroko Noda-Heiny, Satoshi Fujii, and Burton E. Sobel

Local accumulation of plasminogen activator inhibitor-1 (PAI-1) in response to thrombosis has been implicated not only in inhibition of fibrinolysis but also in the pathogenesis of vascular disease. To determine whether thrombin, known to be released from thrombi, can induce expression of PAI-1 in vascular smooth muscle, bovine aortic smooth muscle cells were exposed to highly purified bovine thrombin. Thrombin, in the absence of serum, induced production of PAI-1 by bovine aortic smooth muscle cells in a dose-dependent manner. PAI-1 activity in the conditioned media reached a maximum with 12 nM thrombin. Metabolic labeling with \[^{35}S\]methionine demonstrated that the elaborated PAI-1 was newly synthesized and that it comprised both a cleaved inactive 42-kd form and an uncleaved active 46-kd form. The increase of PAI-1 activity in the media paralleled the thrombin-induced increase in the concentration of the 46-kd form. Preincubation of thrombin with hirudin, a specific inhibitor of thrombin, or with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, an inhibitor of the active site of thrombin, prevented the induction of PAI-1 synthesis. The stimulatory effect of thrombin on PAI-1 synthesis was also evident at the level of expression of mRNA, with steady-state PAI-1 mRNA levels increasing by 100% in 4–8 hours. When the bovine aortic smooth muscle cells were exposed to transforming growth factor-\(\beta_1\), an agonist shown previously to increase PAI-1 synthesis in diverse cell types, synergy with thrombin was evident. Thus, production of PAI-1 by vascular smooth muscle cells is augmented by thrombin, potentially predisposing the cells to persistent thrombi and to vasculopathy at sites of thrombosis and at sites of endothelial injury or denudation.

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KEY WORDS • thrombin • plasminogen activator inhibitor-1 • transforming growth factor-\(\beta_1\) • vasculopathy • smooth muscle cells

Atherosclerosis involves sequential complex phenomena that often follow endothelial injury. Platelet activation and aggregation, thrombosis, and smooth muscle cell proliferation are prominent.\(^1\) Thrombin, a powerful activator of platelets, elicits release of growth factors and chemotactic agents from platelets and intact cells.\(^2\) Its potential contribution to atherosclerosis is suggested by its presence in thrombi and the extracellular matrix (ECM) within evolving plaques.\(^3\)

Abnormally high concentrations of thrombin (60–90 nM) are present in blood when intravascular thrombosis is in progress\(^4,5\) and in thrombi that persist after recanalization has been induced with thrombolytic drugs.\(^6\) The surfaces of residual thrombi are twofold more powerful as stimuli for thrombosis than is the freshly injured arterial wall, partially because of the presence of functionally active thrombin.\(^7,8\) When injury to a vessel wall involves denudation of endothelium and the medial smooth muscle cell layer is either exposed or damaged, atherogenesis is accelerated.\(^9\) Both proliferation and migration of smooth muscle cells become prominent.\(^10\)

Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor (Serpin), is the primary physiological inhibitor of urokinase (u-PA) and tissue-type (t-PA) plasminogen activators.\(^11\) Its importance in modulating fibrinolysis is supported by the correlation of high plasma levels of PAI-1 with an increased incidence of myocardial infarction and deep vein thrombosis.\(^12,13\) In addition, it may play a pivotal role in normal and pathological arterial wall remodeling after injury.\(^15\) Thrombin can modify PAI-1 expression in human endothelial cells\(^16,17\) and in hepatoma (HepG2) cells in culture.\(^18\) However, its potential effects on the production of PAI-1 in vascular smooth muscle cells have not yet been elucidated. Because such cells are exposed to thrombin in blood and in thrombi when deep vessel injury occurs, we undertook the present study to determine whether thrombin can induce synthesis of PAI-1 in vascular smooth muscle cells in culture.

Materials and Methods

**Materials**

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (PS) (30 units/ml, 30 μg/ml), and 10× trypsin-EDTA were obtained from Gibco Laboratories, Grand Island, N.Y. DMEM/Ham's nutrient
were obtained from Sigma Chemical Co., St. Louis, Mo. Mouse monoclonal anti-α-actin antibody and random primer DNA labeling kits were obtained from Boehringer-Mannheim Corp., Indianapolis, Ind. ABC-GO kits and the glucose oxidase stain were obtained from Vector Laboratories, Inc., Burlingame, Calif. Highly purified bovine α-thrombin (3,262 NIH units/ml per milligram) was supplied by Enzyme Research Inc., South Bend, Ind. The chromogenic substrate S-2251 and human Glu-plasminogen were obtained from Kabi Vitrum, Franklin, Ohio. TintElize kits (Biopool, AB, Umea, Sweden) and goat anti-human PAI-1 antibody were acquired from American Diagnostica Inc., Greenwich, Conn. [35S]Methionine and [3H]dCTP were purchased from Amersham Corp., Arlington Heights, Ill.; protein G-sepharose and NAP-5 columns from Pharmacia, Piscataway, N.J.; and RNAzol B from Tel-test, Inc., Friendswood, Tex. d-Phenylalanyl-1-prolyl-l-arginine chloromethyl ketone (PPACK) was obtained from Calbiochem Corp., La Jolla, Calif.; hirudin from Fluka, Buchs, Switzerland; human recombinant insulin-like growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1, purity >95% by 18% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]), platelet-derived growth factor (PDGF), and anti-TGF-β1 antibody from Collaborative Research, Inc., Bedford, Mass.; and rabbit antiserum to bovine and human vitronectin from Telios Pharmaceuticals, Inc., San Diego, Calif. All other reagents were of the highest grade available.

Cell cultures. Bovine aortic smooth muscle cells (BASMCs) were harvested by preparing explants from the aorta of freshly slaughtered cattle as outlined by Ross.19 The cells were cultured initially on 9.6-cm² six-well plates (Falcon, Lincoln Park, N.J.) in DMEM with 1% PS and 10% FBS. To verify the purity of the smooth muscle cell cultures, cells at passage 1 were incubated with mouse anti-α-actin antibody and visualized with fluorescein isothiocyanate–labeled goat anti-mouse IgG or glucose oxidase with the use of ABC-GO kits. Cultured cells were incubated at 37°C and 5% CO2. Subsequent experiments were performed with cells from passages 2–6. To ensure equal cell number in each well per experiment, cells were counted on the hemocytometer, equally plated, and allowed to come to confluence. Human umbilical vein endothelial cells (HUVECs) were obtained as outlined previously.20

Detection of PAI-1 antigen, PAI-1 activity, and t-PA activity in conditioned media. Cells were exposed to highly purified human thrombin in concentrations of 3.0, 6.0, 12, 36, 60, and 120 nM after they had been cultured in DMEM with 1% PS and 10% FBS and allowed to come to confluence in 9.6-cm² wells (1.0 x 10⁵ cells/cm²). Media were removed, and the cells were washed three times with PBS before being placed in serum-free DMEM/F-12 HEPES media (growth arrest media) for 48 hours. Subsequently, aliquots of fresh growth arrest media containing selected concentrations of thrombin were added to the wells. After 24 hours, conditioned media (CM) were collected, supplemented with 0.01% Tween 80, and frozen at −20°C until assay. PAI-1 activity was assayed with the use of S-2251, a chromogenic substrate, by measuring inhibition of activation of plasminogen by t-PA.21 and t-PA activity was assayed by a spectrophotometric solid-phase fibrin-tissue plasminogen activator assay.22 PAI-1 antigen was quantified by enzyme-linked immunosorbent assay (ELISA) (TintElize, Biopool).23 In time-course experiments, cells were exposed to thrombin after the selected growth-arrest interval, and aliquots of CM were collected 4, 8, 12, 16, 24, 32, and 48 hours later.

To define the effects of thrombin on the production of PAI-1 by vascular smooth muscle cells, positive control experiments were performed with TGF-β1 and PDGF, agents known to induce its synthesis. After the growth-arrest period, TGF-β1 at concentrations of 0.1, 0.5, and 1.0 ng/ml or PDGF at 10.0 ng/ml were added to the media. In some experiments, growth factors and thrombin were added simultaneously. In others, thrombin was preincubated with hirudin, a specific inhibitor of thrombin, or with PPACK, an active site inhibitor of Serpins, to determine whether the observed effects of thrombin on PAI-1 synthesis by vascular smooth muscle cells required a functionally intact active site. Quantification of the synthesis of PAI-1 and vitronectin by metabolic labeling and immunoprecipitation. BASMCs were grown to confluence in 9.6-cm² wells and growth-arrested for 48 hours. [35S]Methionine (25 μCi/ml of media) was added at the time of exposure of the cells to thrombin, and aliquots of CM were collected after 24 hours.

ECM was isolated by a modification of the method of Mimuro et al27 as follows: Cells in T-75 flasks were washed three times with PBS containing 0.5% Triton X-100 at 37°C for 10 minutes. After complete disruption of the cells with 25 mM NH4OH for 2 minutes, cellular debris was removed by three washes with PBS. ECM was extracted from material remaining adherent to the flask with 1 ml of 0.1% SDS in PBS.

Kessler's method,24 slightly modified, was used for immunoprecipitation of PAI-1 protein. Briefly, the CM or ECM was supplemented with 0.1% bovine serum albumin and incubated with 5 μl goat anti-PAI-1 polyclonal antibody for 16 hours with agitation at 4°C. Immune complexes were precipitated with 100 μl protein G–Sepharose (50% suspension). The beads were collected, microcentrifuged, washed twice with radioimmunoprecipitation buffer (200 μl PBS, 0.1% SDS, 0.5% Nonidet P-40, and 0.1% sodium deoxycholate), and washed once with PBS. The pellet was resuspended in reducing buffer,25 boiled for 3 minutes, and microcentrifuged briefly. The supernatant fraction (15 μl) was subjected to electrophoresis on 10% polyacrylamide gels; the bands comprising radiolabeled PAI-1 were quantified with an Ambis radioisotopic scanner (Automated Radiomicrobiology Systems, San Diego, Calif.); and autoradiography was performed with Kodak XAR-5 film. The same procedure was repeated for assay of newly synthesized vitronectin with the use of a rabbit anti-bovine vitronectin polyclonal antibody.

To verify the functional integrity of the anti-bovine vitronectin, HepG2 cells were metabolically labeled as above, and newly synthesized vitronectin was immuno-
precipitated by anti-bovine vitronectin as well as anti-human vitronectin.

**Western Blot of Bovine Vitronectin in FBS and in CM of BASMCs**

To confirm the origin of vitronectin in our cell culture, Western blotting of bovine vitronectin was performed for FBS and for a 24-hour sample of BASMC CM after growth arrest for 48 hours. Briefly, proteins contained in both samples were separated by SDS-PAGE in triplicate and transferred onto a nitrocellulose membrane. After an overnight block of nonreacting binding sites with 1× transfer buffer solution (0.025 M Tris, 0.192 M glycine, and 10% methanol) plus 3% bovine serum albumin, each sample was exposed to either 1:100, 1:1,000, or 1:10,000 dilution of stock anti-bovine vitronectin. After a 1-hour wash with transfer buffer solution, the membrane was exposed to \[^{32}P\]goat anti-rabbit IgG. These membranes were again washed for 1 hour, dried briefly, and autoradiographed.

**Effects of thrombin on overall protein synthesis.** To determine whether the effects of thrombin on PAI-1 synthesis were attributable simply to its effects on overall protein synthesis, BASMCs were plated on 9.6-cm² plates, grown to confluence, and growth-arrested for 48 hours. At time 0, media were discarded and replaced with serum-free media containing \[^{35}S\]methionine at a concentration of 1 μCi/ml with or without 60 nM thrombin. Twenty-four hours later, the media were discarded, and the cells were washed three times with PBS and exposed to 10% (wt/vol) trichloroacetic acid at 0°C for 10 minutes followed by 100% ethanol. After drying, the cells were resuspended in 0.4N NaOH, and the extracts were heated at 56°C for 30 minutes. The hydrolysate (200 μl) was added to 10 ml Aquasol-2 (DuPont, Boston) for assay of radioactivity that had been incorporated into protein by liquid scintillation spectrometry.26

**Cellular content of PAI-1 mRNA.** RNA was isolated by the acid guanidium thiocyanate–phenolchloroform method.27 Total cellular RNA (15 μg) was electrophoresed on a 1.5% formaldehyde agarose gel and assayed by Northern blotting.28 Prehybridization with 50% formaldehyde, 10% Denhardt’s solution, 0.5 M Tris-HCl, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA was performed for 16 hours at 42°C. Hybridization was performed for 24 hours with a 0.9-kb \[^{32}P\]cDNA probe generated by digestion of human PAI-1 cDNA with EcoRI and Sal I or with 0.9-kb bovine PAI-1 cDNA generated by digestion of 2.7-kb bovine cDNA with EcoRI and BamHI, isolated by batch affinity absorption with sodium iodide glass beads (Geneclean, BIO 101, Inc., Vista, Calif.) and with a 0.6-kb probe for glyceraldehyde-3-phosphate dehydrogenase (XbaI/HindIII digestion) labeled with \[^{32}P\]dCTP. The radioactivity associated with hybridized bands was quantified by radioisotopic scanning and by autoradiography with Cronex intensifying screens and incubation at −70°C.

**Guanidine Activation of Latent PAI-1 in CM**

Latent PAI-1 was activated as described by Reilly and McFall.29 Briefly, CM were collected, combined with Tween 80 (0.01%), and microcentrifuged to remove cellular debris. The CM were then adjusted to 5.4 M guanidine HCl, 0.05 M Tris, 0.15 M NaCl, and 0.01% Tween 80 and incubated at room temperature for 5 minutes. Activated PAI-1 (500 μl) was collected by passing the treated CM over a NAP-5 column and eluting with 1 ml TNT buffer (0.05 M Tris, 0.15 M NaCl, and 0.01% Tween 80, pH 7.6).

**Assay of Bovine PAI-1 Protein by ELISA**

The correlation between values obtained with TintElize kits (calibrated for detection of human PAI-1) and the actual concentrations of bovine PAI-1 was delineated by standardizing the amount of bovine and human PAI-1 protein with respect to the ratio between PAI-1 activity and PAI-1 antigen for each (TintElize). CM were collected from BASMCs and from HUVECs. Latent PAI-1 was activated with guanidine HCl as described above.30 Immediately after purification of the activated PAI-1 through a NAP-5 column, PAI-1 activity was assayed with the chromogenic substrate S-2251. A total of 10 samples of CM containing bovine PAI-1 with activities between 0 and 72 absorbance units (AU)/ml were analyzed by ELISA. Untreated CM of HUVECs contained 2.27 ng/ml PAI-1 protein, which measured 1 AU/ml on the activity assay (n=3). For the bovine PAI-1, 0.06 ng/ml protein was detected for 1 AU/ml measured on the activity assay. The standard curve was linear throughout the range. Therefore, the measured bovine PAI-1 concentration was multiplied by 40 (factor defined for sensitivity of detection of bovine PAI-1 protein) to estimate the true concentration of bovine PAI-1 protein in CM and extracts.

**Statistical Analysis**

Statistical analysis was performed on the present data using either Student’s t test or analysis of variance, and p<0.05 was taken as significant unless otherwise stated.

**Results**

**Effects of Thrombin on PAI-1 Antigen, PAI-1 Activity, and t-PA Activity in CM of BASMCs**

The concentration of PAI-1 antigen was measured in CM after stimulation of BASMCs with thrombin at a concentration of 60 nM (5 NIH units/ml media), insulin at 8.7 nM, IGF-1 at 84.4 nM, and a combination of insulin and IGF-1. The concentration of thrombin that produces maximal proliferation of vascular smooth muscle cells was the one selected for evaluation of effects on the production of PAI-1.30 The concentrations of insulin and IGF-1 selected were those we have previously shown to induce maximal stimulation of PAI-1 synthesis by cultured HepG2 cells.31 CM were collected at selected intervals as shown in Figure 1. Within 8 hours, thrombin had exerted a potent stimulatory effect on PAI-1 synthesis, as reflected by an increase in the concentration of PAI-1 in CM to 418 ng/ml as compared with values of 54, 202, and 92 ng/ml after stimulation with insulin, IGF-1, and the combination of insulin and IGF-1, respectively. In the absence of any of the agonists, no PAI-1 protein was detectable.

The BASMCs were exposed to thrombin concentrations between 0 and 120 nM to define the concentration–response curve, with the recognition that at high concentrations thrombin may affect the persistence of
PAI-1 protein or activity, directly or indirectly, independent of effects on synthesis. The higher concentrations of thrombin resulted in elaboration of more detectable PAI-1 protein with a 50% effective concentration \((EC_{50})\) occurring at 3 nM (Figure 2, top panel). However, PAI-1 activity was maximal after exposure of the cells to 12 nM thrombin, as shown in Figure 2, bottom panel. Thus, the \(EC_{50}\) for thrombin is between 3 and 6 nM. Thrombin at 1.5 nM exerted no effect. t-PA activity in all samples measured was zero. However, t-PA antigen could not be measured with the ELISA used (American Diagnostica), because the antibody did not recognize bovine t-PA. Activation of latent PAI-1 by guanidine HCl yielded a 10–20-fold increase in activity. Thus, the bulk of PAI-1 protein secreted was latent PAI-1 in CM from both control and thrombin-stimulated cells.

**Immunoprecipitation of Newly Synthesized PAI-1**

To determine whether thrombin stimulated PAI-1 protein synthesis or whether it simply released preformed PAI-1 from the ECM, metabolic labeling was implemented with \(^{35}S\)methionine added to the media at the time of exposure of the cells to thrombin. CM, harvested after 24 hours, were supplemented with anti-PAI-1 antibody. Autoradiography was used to detect the immunoprecipitated, metabolically labeled, and hence newly synthesized PAI-1. It revealed two distinct bands (42 and 46 kd) after exposure of the cells to each concentration of thrombin. Whereas the total amount of labeled PAI-1 (the 42-kd and the 46-kd material) increased monotonically with increasing concentrations of thrombin, the concentration of the 42-kd (cleaved) PAI-1 increased at high concentrations of thrombin (Figure 3), but the concentration of the 46-kd PAI-1 did not. Furthermore, PAI-1 activity correlated with the concentration of the 46-kd (uncleaved) newly synthesized PAI-1 species.

When the same procedures were performed with antivitronectin antibody instead of anti-PAI-1, no radioactively labeled precipitates were obtainable (in contrast to the case with metabolically labeled vitronectin synthesized by HepG2 cells that reacted with the antibody and served as a positive control). From the results of the Western blot, the origin of the vitronectin in the CM appears to be the FBS added to the media during the growth phase of the cells. These results indicate that thrombin induced synthesis of PAI-1 without inducing synthesis of vitronectin and that high concentrations of thrombin in CM resulted in cleavage and some inactivation of PAI-1.

**Effects of Thrombin on Overall Protein Synthesis in BASMCs**

After exposure to either serum or thrombin, growth-arrested BASMCs enter the cell cycle with maximal DNA synthesis occurring in the next 18–24 hours, as determined by \(^{3}H\)thymidine incorporation and flow
cytometry. Accordingly, the prompt increase in PAI-1 protein we observed in the present study cannot be attributed simply to an increase in the number of the BASMCs producing protein. However, the increase in PAI-1 could be a reflection of nonspecific increases in overall protein synthesis. To evaluate this possibility, [35S]methionine incorporation into total protein was measured by assay of radioactivity in trichloroacetic acid (10% [wt/vol])–precipitable protein. Thrombin did not increase total protein synthesis significantly over 24 hours (n = 3, p > 0.05 by Student’s t test), nor did it alter the amount of [35S]methionine–labeled PAI-1 protein appearing in and extractable from the ECM (n = 3, p > 0.05 by Student’s t test). Thus, its effects on PAI-1 synthesis and elaboration into the CM appear to be specific.

Effects of Thrombin on Steady-State Concentrations of PAI-1 mRNA

To determine whether thrombin modified the expression of PAI-1 mRNA, thereby accounting for the increased synthesis of PAI-1 protein, total cellular RNA harvested from cells exposed to thrombin was subjected to Northern blotting with a PAI-1 probe. A GAP probe, as well as the visualization of 18S rRNA with ethidium bromide staining, was used as a positive control. Because of the similarity in molecular weights of the minor PAI-1 species (1.6 kb) and glyceraldehyde-3-phosphate dehydrogenase mRNA (1.5 kb), initially hybridized membranes were stripped and rehybridized with PAI-1 probe alone. The 3.0-kb PAI-1 mRNA levels increased compared with those in control cells within 4 hours and peaked between 4 and 8 hours, with an increase of approximately 100% (Figure 4). The 1.6-kb PAI-1 mRNA band was found to contain between 10% and 20% of the total PAI-1 mRNA detected (3.0+1.6 kb). Its concentration increased proportionally with that of the 3.0-kb form. The increase in PAI-1 mRNA is consistent with the view that thrombin augments PAI-1 synthesis at the level of expression of mRNA by increasing transcription, stabilizing PAI-1 transcripts, or both.

Synergistic Effects of Thrombin and Selected Agonists on Production of PAI-1

To determine whether the effects of thrombin were additive or synergistic with those of known stimuli of production of PAI-1 in BASMCs, experiments were
performed with PDGF (10.0 ng/ml) and TGF-β1 (0.5, 1.0, and 10.0 ng/ml) (Figure 5). In contrast to results of studies in which serum was used in CM, neither PDGF nor TGF-β1 alone, in the absence of serum, significantly stimulated production of PAI-1 in the BASMCs. However, when thrombin (60 nM) was added to serum-free media containing TGF-β1, a significant increase in production of PAI-1 occurred that exceeded that attributable simply to additive effects (Figure 6). Incubation of cells with thrombin plus anti-TGF-β antibodies did not abolish the stimulatory effect of thrombin, indicating that the thrombin effect is not mediated by extracellular TGF-β1.

**FIGURE 5.** Bar graph showing plasminogen activator inhibitor-1 (PAI-1) protein in conditioned media of bovine aortic smooth muscle cells exposed to selected agonists for 24 hours with and without 5% fetal bovine serum (FBS). Agonists include 60 nM thrombin, 10.0 ng/ml platelet-derived growth factor (PDGF), and 1.0 and 10.0 ng/ml transforming growth factor-β1 (TGF-β). There was no significant difference between control and each of the agonists when 5% FBS was added. In serum-free media, only the difference between control and thrombin 60 nM was statistically significant (*p<0.0001) by two-way analysis of variance.

**FIGURE 6.** Bar graph showing synergistic effects of thrombin and transforming growth factor-β1 (TGF-β) on the production of plasminogen activator inhibitor-1 (PAI-1) protein by bovine aortic smooth muscle cells. Increasing concentrations of TGF-β were added to media containing 60 nM thrombin.

**FIGURE 7.** Bar graph showing relative concentration of plasminogen activator inhibitor-1 (PAI-1) protein detected in conditioned media of bovine aortic smooth muscle cells after preincubation of thrombin with thrombin inhibitors, t-phenyl-alanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, 600 nM), and hirudin (120 nM). Hirudin at the same concentration was added to 5% media containing fetal bovine serum (FBS). Each separate experiment was standardized to its own control (i.e., results with serum-free conditioned media alone) designated as an arbitrary unit. Results from multiple experiments are shown (mean ±SEM). There were no significant differences between control (serum-free media only) and thrombin plus PPACK (PPACK+Thr) or hirudin (hirudin+Thr). Thrombin exerted statistically significant effects compared with control media (*p<0.05 by analysis of variance with Bonferroni’s correction). Results with hirudin with media containing 5% FBS were statistically different from those with control media with 5% FBS (†p<0.05 by analysis of variance with Bonferroni’s correction).

**Importance of the Active Site**

Preincubation of thrombin with an active site inhibitor, PPACK, abolished the stimulatory effect of thrombin on production of PAI-1. Hirudin, another thrombin inhibitor, which blocks both the active site and the anion binding exosite, abolished the effect as well. When hirudin was added to serum, the stimulatory effects of serum on PAI-1 appeared to be somewhat diminished (Figure 7). Thus, thrombin or a thrombin-like substance in blood may influence regulation of PAI-1 synthesis in vivo.

**Discussion**

Results of the present study indicate that thrombin increases synthesis of PAI-1 by vascular smooth muscle cells at concentrations similar to those impinging on the media of vessels with deep injury or endothelial denudation in vivo. Compared with other agonists, including IGF-1, TGF-β, PDGF, and insulin, thrombin was particularly powerful. In contrast to the case with PDGF and TGF-β, thrombin stimulated production of PAI-1 in vascular smooth muscle cells in the absence of serum. Since PDGF has been shown to stimulate neointimal accumulation after angioplasty in athymic nude rats,32 potentiation of PAI-1 production by thrombin and PDGF may also play a role in its rate of accumulation.

PAI-1 is associated with the ECM of vascular smooth muscle cells in culture.33,34 However, thrombin did not
stimulate the release of newly synthesized PAI-1 from the ECM. Results of the immunoprecipitation studies with antivitronectin antibodies showed that no new vitronectin was being synthesized by BASMCs. Because the cells were studied in serum-free media, the CM was devoid of vitronectin as well. Accordingly, the increase in PAI-1 was not secondary to elaboration of vitronectin or stabilization of elaborated PAI-1 by vitronectin in BASMCs stimulated with thrombin.

Thrombin can cleave the carboxyl terminus of PAI-1, thereby inactivating it. Accordingly, it is not surprising that PAI-1 with the highest specific inhibitory activity was harvested from CM of cells exposed to only modest amounts (12 nM) of thrombin. At higher concentrations, 60 and 120 nM, thrombin continued to augment the net production of PAI-1. However, the cleaved and inactive 42-kd protein composed an increasing fraction of the total. Thus, the total amount of PAI-1 increased monotonically with increasing concentrations of thrombin. These results suggest that transitory exposure of vascular smooth muscle cells to thrombin, such as that associated with local clot formation in vivo, would result in elaboration of active PAI-1. The 60–90-nM concentration range of thrombin studied conforms to the range estimated to be present locally in vivo.

Although thrombin exhibits mitogenic properties, activates protein kinase C, and stimulates protein synthesis, the increases observed in PAI-1 mRNA occurred within 4 hours of exposure of the cells to thrombin after growth arrest for 48 hours. The increases occurred much earlier than the augmentation of DNA synthesis inducible by thrombin in 18–24 hours. Accordingly, the effect of thrombin on PAI-1 synthesis is not attributable to either cellular proliferation or a nonspecific stimulation of overall protein synthesis (as judged from the results of our experiments with trichloroacetic acid precipitation). An active site does appear to be required, consistent with observations in cultured HUVECs, in which PAI-1 synthesis is increased by thrombin but pretreatment of the thrombin with diisopropylfluorophosphate or hirudin abolishes the effect.

The pathophysiologic implications of augmentation of PAI-1 synthesis by thrombin in vascular smooth muscle cells have not yet been elucidated. However, we have shown that thrombin, as well as other agonists, are capable of affecting BASMC production of PAI-1 independent of the overlying endothelium. Perhaps this further decreases fibrinolytic activity and enhances hemostasis in deep vessel injuries, or perhaps PAI-1 has another role in the vascular smooth muscle cell that is related to neointimal formation. Because PAI-1 activity as well as protein increased and because plasminogen-activating activity in the same samples was zero, our results indicate that the elaboration of PAI-1 is not accompanied by a parallel elaboration of u-PA, t-PA, or both, which would have complexed with PAI-1 and attenuated its assayable activity.

It seems likely that the augmentation of PAI-1 synthesis in vascular smooth muscle cells exposed to thrombin may exacerbate occlusive vascular disease through several mechanisms. Elaboration of PAI-1 from vascular smooth muscle cells exposed to blood and thrombin in vivo as a result of endothelial injury or denudation may predispose them to the persistent thrombi or to recurrent thrombosis as a result of local inhibition of fibrinolysis. In addition, it may exacerbate occlusive vascular disease by altering repair and remodeling of vessels with complex plaques, fissures, or deep injury such as that seen with angioplasty. Vascular PAI-1 has been implicated in attenuating cell migration otherwise induced by plasminogen activators such as u-PA within the vessel wall as well as in stabilizing thrombi. Consequently, its augmented synthesis may predispose the vessel to bombardment by mitogens associated with platelets and other constituents of thrombi and to impaired remodeling, which potentially intensifies restenosis after thrombolysis, angioplasty, or both. Recent observations implicating increased PAI-1 in blood in patients with restenosis after angioplasty and in vessel walls undergoing remodeling in regions juxtaposed to thrombi in carotid arteries of experimental animals are consistent with this possibility.

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