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Inhibitory Effect of a New Butadiene Derivative on the Production of Plasminogen Activator Inhibitor-1 in Cultured Bovine Endothelial Cells

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Tissue-type plasminogen activator (t-PA) and its physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1), are known to be synthesized by vascular endothelial cells and to play important roles in regulating the fibrinolytic activity of plasma. We found that a new butadiene derivative, (3*E*,4*E*)-3-benzylidene-4-(3,4,5-trimethoxybenzylidene)pyrrolidine-2,5-dione (T-686), inhibits PAI-1 production without affecting plasminogen activator (PA) synthesis in cultured bovine endothelial cells. T-686 (1-10 μM) dose-dependently decreased the accumulation of PAI-1 in conditioned medium from the treated cells and elevated PA activity in the conditioned medium. Analysis of the conditioned medium by the zymography technique indicated that T-686 decreased the activities of PAI-1 with an M_r of 55,000 and t-PA/PAI-1 complex with an M_r of 99,000. Furthermore, T-686 attenuated the augmentation of PAI-1 antigen induced by lipopolysaccharide in the conditioned medium. The decrease of PAI-1 antigen was in parallel with the reduction of the PAI-1 mRNA level (Northern blots). These results suggest that T-686 can promote net fibrinolytic activity through suppression of PAI-1 production without affecting PA elaboration in endothelial cells.

Key words: endothelial cell, fibrinolysis, plasminogen activator, plasminogen activator inhibitor-1, T-686.

Tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) activate the fibrinolytic system by converting the inactive zymogen, plasminogen, into the active proteolytic enzyme, plasmin. The net fibrinolytic activity in blood is mainly determined by the balance between PA and plasminogen activator inhibitor-1 (PAI-1) (*1-3*). The importance of PAI-1 in regulating this balance is emphasized by a variety of clinical studies: a deficiency and decreased functional activity of PAI-1 are responsible for a life-long bleeding disorder (*4, 5*) and increased concentrations of PAI-1 in plasma are associated with diverse thrombotic diseases, including deep venous thrombosis (*6-8*) and coronary artery disease (*9-11*). Therefore, inhibition of PAI-1 activity or reduction of PAI-1 production by a pharmaceutical agent may be useful for prevention and/or treatment of thrombotic disorders.

Since vascular endothelial cells synthesize and secrete both t-PA and PAI-1, cultured endothelial cells are suitable for the study of the modulation of endogenous fibrinolytic activity in an *in vitro* system (*12*). In cultured endothelial cells, physiological substances such as thrombin (*13, 14*), short-chain fatty acids (*15*), and retinoids (*16, 17*) have been shown to increase t-PA synthesis, while lipopolysaccharide (LPS) or cytokines such as IL-1β, TGF-β, and TNF-α induce PAI-1 production by endothelial cells (*18-21*). Recently, it has been shown that a fibrate compound, gemfibrozil, inhibits expression of PAI-1 *in vitro* and *in vivo* (*22, 23*).

In this paper, we report the identification and characterization of a novel inhibitor of PAI-1, a new butadiene derivative {T-686, (3*E*,4*E*)-3-benzylidene-4-(3,4,5-trimethoxybenzylidene)pyrrolidine-2,5-dione} (Fig. 1). This compound, discovered through random screening of synthesized compounds, inhibits the augmentation of LPS-induced PAI-1 synthesis as well as basal PAI-1 production, but does not affect PA production in cultured bovine endothelial cells.

MATERIALS AND METHODS

Materials—T-686 was synthesized at the Lead Optimization Research Laboratory of our company. Bovine fibrinogen (90% clottable), human plasminogen (20 CU), and lipopolysaccharide (LPS, *Escherichia coli*; 0111: B4) were purchased from Sigma Chemical (St. Louis, MO); Fetal calf serum (FCS) and L-glutamine from GIBCO (Grand Island, NY); Eagle's minimum essential medium (E'MEM) from Nissui Seiyaku (Tokyo); and Triton X-100 from Nacalai Tesque (Kyoto). A kit for determination of plasminogen activator (PA) activity (Spectrolyse™/fibrin) was obtained from Biopool AB (Umea, Sweden). A Fast

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Abbreviations: BCaEs, bovine carotid artery endothelial cells; FA, fibrin autography; FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate-dehydrogenase; LPS, lipopolysaccharide; MEM, minimum essential medium; PA, plasminogen activator; PAGE, polyacrylamide gel electrophoresis; PAI-1, plasminogen activator inhibitor-1; RFA, reverse fibrin autography; t-PA, tissue-type plasminogen activator; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

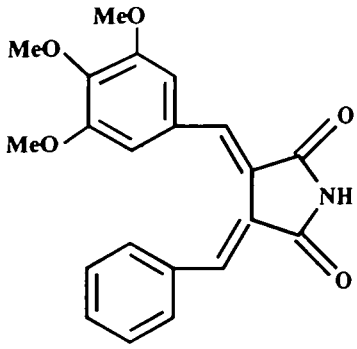

Fig. 1. **Chemical structure of T-686, (3*E*,4*E*)-3-benzylidene-4-(3,4,5-trimethoxy-benzylidene)pyrrolidine-2,5-dione.**

Track mRNA isolation kit was purchased from Invitrogen (San Diego, CA); a Geneclean II kit from B10 101 (LaJolla, CA); a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) from CLONTECH (Palo Alto, CA); [^{32}P]dCTP from Amersham (Arlington Heights); a Random Primer DNA Labeling kit from Takara Shuzo (Shiga); BIODYNE B nylon membrane from Pall BioSupport (Glen Cove, NY); and Fuji medical X-ray film (New RX) from Fuji Film (Tokyo). All other reagents were of the highest grade available.

Endothelial Cell Culture—Bovine carotid artery endothelial cells (BCaEs) were isolated from carotid arteries with a scalpel by the technique described by Gospodarowicz *et al.* (*24*), and the cells were cultured in flasks in E'MEM supplemented with 10% FCS and 2 mM L-glutamine. In the present study, BCaEs at passages 9 through 20 were used.

Preparation of Conditioned Medium—BCaEs were grown to confluency in 24- or 6-well microplates. The cell monolayer was washed with FCS-free MEM and incubated for 24 h with FCS-free MEM. The medium was removed and replaced with fresh FCS-free MEM in the presence or absence of T-686 dissolved in 0.5% ethanol (final concentration). Control cells were incubated with medium containing 0.5% ethanol only. After incubation for 24 h, the conditioned medium was collected and centrifuged at 1,000×*g* for 10 min at 4°C to remove cellular debris. The conditioned medium was stored at −80°C until measurement of PAI-1 antigen, and determination of PA activity.

Fibrin Autography (FA) and Reverse Fibrin Autography (RFA)—BCaEs were treated with T-686 for 24 h, then an aliquot of the conditioned medium was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (*25*). Its PA and PAI-1 activities on the polyacrylamide gel were analyzed by FA and RFA (*26*). The SDS was removed by incubating the polyacrylamide gel with 2.5% Triton X-100 in 0.05 M Tris-HCl, 0.15 M NaCl (pH 7.5). In FA, the gel was placed on fibrin-agar indicator gel consisting of 1% agarose, 2 mg/ml fibrinogen, 0.6 U/ml thrombin, and incubated at 37°C for 4.5 h. In RFA, the polyacrylamide gel was incubated at 37°C for 2 h on the indicator gel, to which 0.13 IU/ml t-PA and 0.03 CU/ml plasminogen had been added. Development of opaque, lysis-resistant zones in the otherwise clear indicator gel reveals PAI-1 activity on the gel.

Effect of T-686 on Elevation of PAI-1 Antigen Induced by LPS in BCaEs—BCaEs were grown to confluency in 75 cm^2 culture flasks, then washed twice with FCS-free MEM, and further incubated for 24 h in FCS-free MEM. The medium was removed and replaced with a medium containing T-686 (1-10 μM) and LPS (3 μg/ml). After incubation for 24 h, the conditioned medium was collected, centrifuged at 3,000 rpm for 10 min at 4°C to remove cellular debris, and stored at −80°C until measurement of PAI-1 antigen. To isolate total cellular RNA, the cells were washed twice in ice-cold phosphate-buffered saline, and collected into a pellet by centrifugation at 1,000×*g* for 10 min at 4°C.

Preparation of DNA Probes—Messenger RNAs (mRNAs) were isolated from HTC rat hepatoma cells by utilizing a Fast Track mRNA isolation kit. A cDNA fragment encoding the full length of rat PAI-1, corresponding to bases 1-1209 of the rat PAI-1 cDNA (*27*), was amplified by the reverse transcription-polymerase chain reaction method. The length of amplified cDNA fragment was determined by agarose gel electrophoresis, and the fragment was purified with a GENECLEAN II kit. The purified fragment was subcloned into the *Hin*dIII site in the pBluescript II SK(+) phagemid vector. The sequence of the cloned cDNA insert was determined by DNA sequencing. The entire cDNA insert for rat PAI-1 and the cDNA fragment for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were labeled with [^{32}P]dCTP by utilizing a Random Primer DNA Labeling kit.

RNA Isolation and Northern Blot Analysis—Total cytoplasmic RNA was isolated by the acid guanidine thiocyanate-phenol-chloroform method (*28*) and quantified by measurement of the absorbance at 260 nm. Agarose gel electrophoresis was performed by the glyoxal method (*29*). Total RNA was denatured with 1 M glyoxal-50% dimethyl sulfoxide, and 15 mg of it was fractionated on 1% agarose gel with 0.1 M phosphate buffer (pH 7.4) under constant voltage (50 V). RNA was transferred from the gel to a BIODYNE B nylon membrane according to the manufacturer's instructions. The membrane was prehybridized at 42°C for 24 h and then hybridized with the radiolabeled cDNA probe for rat PAI-1 and for human G3PDH in a hybridization buffer [50 mM sodium phosphate, pH 6.8, 50% formamide, 5×saline-sodium-citrate, 5×Denhardt's solution, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast t-RNA] at 42°C for 16 h. After hybridization, the membrane was washed twice with 0.2×saline-sodium-citrate, 0.1% SDS at 42°C for 30 min. Autoradiography was performed at −80°C with a Fuji medical X-ray film. The radioactivity associated with hybridized bands was determined by using a bioimage analyzer, BAS2000 (Fuji Film).

Assays—PA activity was determined by the spectrophotometric method (*30*) using a commercial kit (Spectrolyse™/fibrin). PAI-1 antigen in the conditioned medium was determined using a specific enzyme-linked immunosorbent assay (ELISA) kit obtained from Biopool AB (*31, 32*). The assay of PAI-1 antigen was carried out in accordance with the Biopool AB protocol.

Statistical Analysis—Data were expressed as means± SE. ANOVA was used for statistical analysis. A difference with $p<0.05$ was considered statistically significant.

RESULTS

Effect of T-686 on Accumulation of PAI-1 Antigen and Net PA Activity in Conditioned Medium from BCaEs—

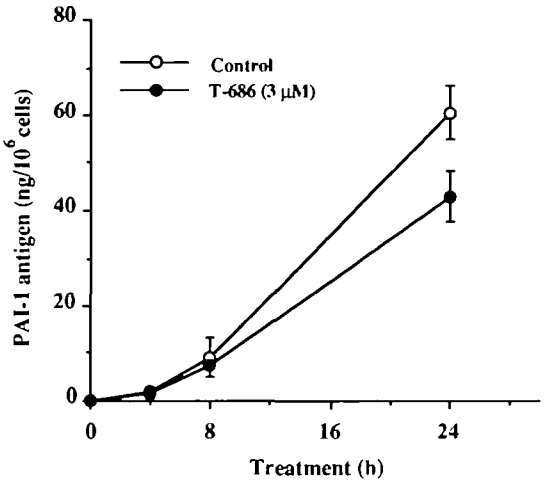

Fig. 2. **Effects of T-686 on secretion of PAI-1 antigen in the conditioned medium of BCaEs.** BCaEs grown to confluency were serum-starved for 24 h and then incubated with FCS-free MEM in the presence or absence of T-686 (3 μM) for the indicated periods of time. After the incubation, the conditioned medium was collected and PAI-1 antigen was determined by ELISA. Values are the means ± SE from triplicate cultures.

PAI-1 antigen was measured in the conditioned medium from BCaEs after treatment with T-686 for 4, 8, and 24 h. Basal secretion of PAI-1 into the conditioned medium for 24 h was 60.5 ± 5.6 ng/10^6 cells (Fig. 2). The basal secretion of PAI-1 was depressed by the treatment with T-686 (3 μM) to about 70% of the control at 24 h. Moreover, the treatment with T-686 (3 and 10 μM) showed significant reductions of PAI-1 antigen in the conditioned medium (Fig. 3B), while the net PA activity was conversely increased (Fig. 3A). Aliquots of the CM obtained from T-686-treated or non-treated cells were fractionated by SDS-PAGE, and analyzed by FA and RFA. In RFA, the activity of PAI-1 with an M_r of 55,000 was dose-dependently reduced by the treatment with T-686 (Fig. 4B). On the other hand, the activity of PA with an M_r of 99,000 was identified by FA (Fig. 4A). The 99 kDa band seems to correspond to t-PA/PAI-1 complex (*33*). The lysis area was decreased by the treatment with T-686 (Fig. 4A). This suggests that the reduction in PAI-1 after the treatment with T-686 results in a decrease of t-PA/PAI-1 complex. Further development in FA (incubated for 30 h), caused the activity of PA having the same M_r of 71,000 as authentic t-PA to appear. However, the activity of PA with an M_r of 71,000 was not affected by the treatment with T-686 (data not shown). These results suggest that T-686 inhibits production and/or secretion of PAI-1 without affecting the

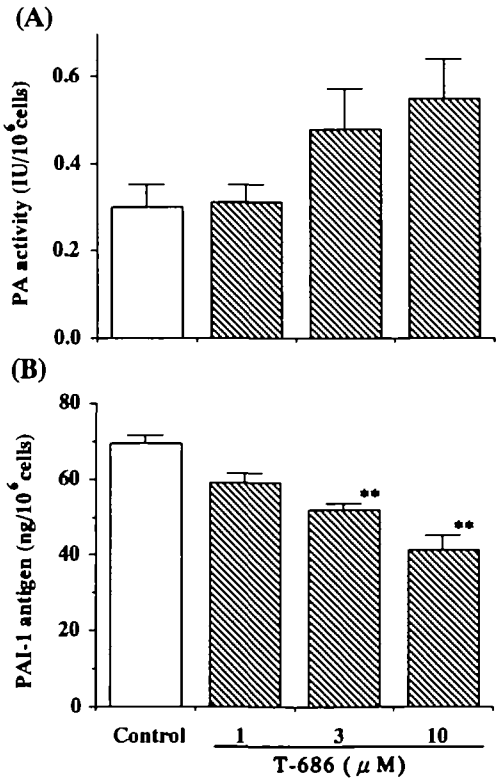

Fig. 3. **Effects of T-686 treatment on the net PA activity and basal secretion of PAI-1 in the conditioned medium from BCaEs.** Confluent BCaEs were serum-starved for 24 h and further incubated with FCS-free MEM in the presence or absence of T-686 (1, 3, and 10 μM) for 24 h. After the incubation, the conditioned medium was collected and PA activity (A) and PAI-1 antigen (B) were determined. Values are the means ± SE from triplicate or quadruplicate cultures. $^{**}p < 0.01$ compared with control.

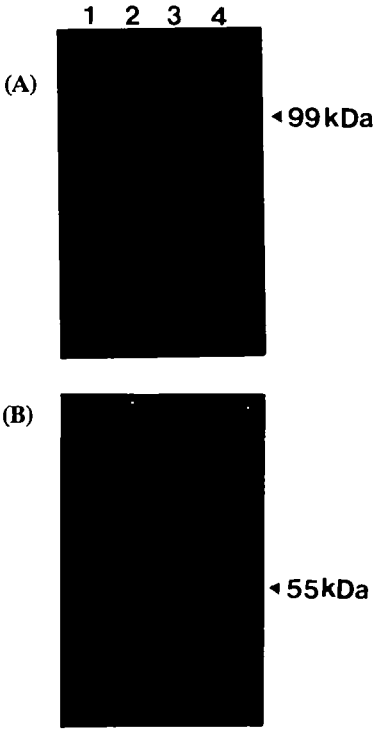

Fig. 4. **Representative zymographs showing the effects of T-686 treatment on secretion of PA and PAI-1 from BCaEs.** Confluent BCaEs were serum-starved for 24 h and further incubated with FCS-free MEM in the presence or absence of T-686. The conditioned medium was collected and 20 μl aliquots were fractionated by SDS-PAGE. The electrophoresed gels were analyzed for PA activity by FA (A) and PAI-1 activity by RFA (B) as described in "MATERIALS AND METHODS." Lanes 1, 2, 3, and 4 show the conditioned medium from BCaEs treated with vehicle alone, 1, 3, and 10 μM T-686, respectively. Molecular weight values (kDa) are indicated on the right.

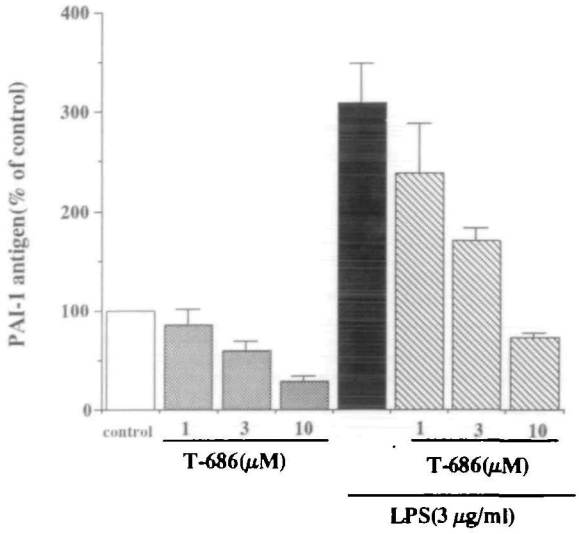

Fig. 5. **Effects of T-686 on the LPS-induced increase of PAI-1 antigen in the conditioned medium from BCaEs.** Confluent BCaEs were incubated in FCS-free MEM for 24 h and then incubated in fresh FCS-free MEM with T-686 (1, 3, and 10 μM) in the presence or absence of LPS (3 μg/ml) for a further 24 h. After the incubation, the conditioned medium was collected and assayed for PAI-1 antigen. Results were expressed as percentage of the control (control value of the vehicle alone was 413.4±62.8 ng/10^7 cells). Values are the means±SE from three separate experiments.

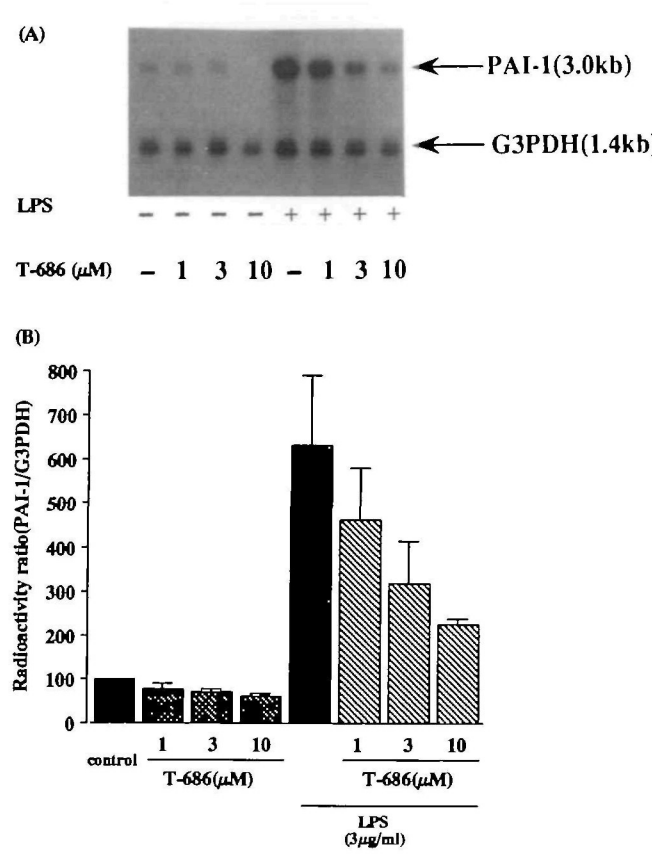

Fig. 6. **Effects of T-686 on PAI-1 mRNA.** (A) A representative autoradiogram of Northern blots of PAI-1 and G3PDH mRNA. BCaEs were serum-starved for 24 h and then incubated with T-686 in the presence or absence of LPS for 24 h as described in the legend to Fig. 5. After the incubation, total cellular RNA was isolated from the BCaEs, and hybridized to rat PAI-1 and human G3PDH cDNA probes. (B) Relative intensities of PAI-1 mRNA to G3PDH mRNA on the radiogram. PAI-1 mRNA levels were determined with a BAS2000 image analyzer and expressed as the ratio of PAI-1 to G3PDH levels (control value of the vehicle alone was 1.04±0.03). Values are the means±SE from three separate experiments.

PA level. Thus, T-686 can enhance the net PA activity when PAI-1 coexists with PA, as shown in Fig. 3A.

Effects of T-686 on the LPS-Induced Increases of PAI-1 Antigen and PAI-1 mRNA in BCaEs—When BCaEs were incubated with 3 μg/ml of LPS for 24 h, PAI-1 antigen in the conditioned medium was markedly increased, as shown in Fig. 5. T-686 (1, 3, and 10 μM) dose-dependently attenuated the LPS-induced PAI-1 antigen (Fig. 5). To determine whether the inhibition of PAI-1 production by T-686 reflected decreased steady-state levels of PAI-1 mRNA in the T-686-treated BCaEs, Northern blots were prepared. Twenty-four hours after incubation with T-686, PAI-1 mRNA levels were decreased dose-dependently (Fig. 6). LPS induced an increase in PAI-1 mRNA levels in the BCaEs in 24 h. T-686 markedly attenuated the LPS-induced augmentation of PAI-1 mRNA without affecting the expression of G3PDH mRNA. Thus, the decrease in PAI-1 synthesis by T-686 was well correlated with the decrease in the steady-state level of PAI-1 mRNA in the BCaEs.

DISCUSSION

A number of etiological studies have indicated that patients with thrombotic diseases have impaired fibrinolytic capacity (*3, 7, 9*). Impaired t-PA release from vascular walls or high PAI-1 levels in plasma have been reported to be responsible for the defective fibrinolysis (*6, 8, 10*). A possible causal relation between the high PAI-1 levels and occurrence of thrombosis may be inferred from the observation that transgenic mice overexpressing human PAI-1 gene suffer from spontaneous thrombosis in the veins of the tail and hind legs (*34*). Moreover, disruption of the PAI-1 gene in mice induces mild hyperfibrinolysis and marked resistance to venous thrombosis (*35*). Thus, drugs that decrease abnormally high levels of PAI-1 in plasma may be useful for improvement of the balance between t-PA and PAI-1 in patients with impaired fibrinolytic activity.

Our results indicate that T-686 inhibits the basal PAI-1 production and markedly attenuates the LPS-induced augmentation of PAI-1 synthesis in cultured bovine carotid artery endothelial cells. Furthermore, the attenuation reflected a parallel decrease in the concentration of PAI-1 mRNA (Fig. 6).

Most of the available information concerning PAI-1 gene expression has been obtained by using cultured cells. In these *in vitro* studies, especially with endothelial cells, it was indicated that LPS, TGF-β, and TNF-α increase PAI-1 synthesis by increasing gene transcription. Induction of PAI-1 mRNA by these agents was not blocked by the protein synthesis inhibitor cycloheximide, suggesting that these events are reflecting signal transduction mechanisms independent of *de novo* protein synthesis (*20*). In addition, it has been reported that down-regulation of protein kinase C levels by phorbol myristate acetate pretreatment in bovine aortic endothelial cells partially attenuated the

response of PAI-1 induction to LPS, but had no effect on the response to TNF-α or TGF-β (*36*). T-686 inhibits the TGF-β-induced increase of PAI-1 production in cultured BCaEs and human umbilical vein endothelial cells (unpublished observation). This suggests that T-686 does not directly suppress the action of LPS and the protein kinase C pathway.

Analysis by fibrin zymography revealed that T-686 dose-dependently reduced the activity of PAI-1 with an M_r of 55,000. This was accompanied by a reduction of the 99 lysis area corresponding to t-PA/PAI-1 complex (Fig. 4). The net PA activity in the conditioned medium of T-686 treated cells was observed to rise when assayed spectrophotometrically with a chromogenic substrate (Fig. 3A). The apparent increase in PA activity probably reflects a decreased amount of PAI-1 in the conditioned medium through inhibition of PAI-1 synthesis by T-686. Therefore, our results indicate that T-686 could promote fibrinolytic activity by suppressing PAI-1 synthesis without affecting the production and/or secretion of PA in the endothelial cells. This possibility was supported by the observation that the content of t-PA antigen was not affected by treatment with T-686 in the conditioned medium of human umbilical vein endothelial cells, although the content of PAI-1 antigen was lowered by the same treatment (unpublished observation). However, studies at the t-PA mRNA level are needed to define unambiguously the effect of T-686 on t-PA production.

As shown in the present study, the action of T-686 had more specificity for PAI-1 production than PA production. The reason for this specificity is not clear yet. Because the protein kinase C pathway plays an important role in regulating t-PA expression (*37*), T-686 may not affect the protein kinase C pathway directly, as discussed above for PAI-1. The mechanisms of the reduction of PAI-1 mRNA expression by T-686 remain to be defined at the level of signal transduction pathways.

Further studies are in progress to investigate whether T-686 can enhance fibrinolytic activity *in vivo* when administered to animals and result in prevention of thrombus formation in experimental thrombosis models.

In conclusion, the present data suggest that T-686 may shift the balance between thrombosis and thrombolysis towards thrombolysis by suppressing PAI-1 synthesis in the vascular endothelium, and may therefore be useful for prevention and/or treatment of thrombotic diseases.

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