VOL. 52 NO. 9, SEPT. 1999 THE JOURNAL OF ANTIBIOTICS pp. 797 — 802

Inhibition of Plasminogen Activator Inhibitor-1 by 11-Keto-9(*E*),12(*E*)-octadecadienoic Acid, a Novel Fatty Acid Produced by *Trichoderma* sp.

TOSHIHIRO CHIKANISHI, CHIKARA SHINOHARA, TADASHI KIKUCHI, AKIRA ENDO and KEIJI HASUMI*

Department of Applied Biological Science, Tokyo Noko University,
Fuchu, Tokyo 183-8509, Japan

We have recently found a novel fatty acid, 11-keto-9(*E*),12(*E*)-octadecadienoic acid (KOD), that enhances fibrinolytic activity of endothelial cells. The mechanism of action of KOD has been investigated. KOD increased 2-fold the plasmin activity of bovine aortic endothelial cells at 250 μM. The stimulation was dependent on plasminogen and was inhibited by anti-urokinase, whereas KOD did not enhance the urokinase-catalyzed plasminogen activation and the resulting plasmin activity in a cell-free system. Neither the production of urokinase nor the conversion of pro-urokinase to the active, two-chain form was elevated by KOD, but it decreased plasminogen activator inhibitor-1 (PAI-1) activity of cells and inactivated PAI-1 irreversibly in a purified system. These results demonstrated that the KOD enhancement of endothelial fibrinolytic activity was mediated, at least in part, by inactivation of PAI-1.

Endothelial cells synthesize and secrete plasminogen activators, such as urokinase (uPA) and tissue plasminogen activator (tPA), as well as plasminogen activator inhibitors. The balance of production between plasminogen activators and inhibitors by endothelial cells mainly regulates fibrinolysis in the blood vessel. In patients with atherosclerosis and thromboembolism, plasminogen activator inhibitor type 1 (PAI-1) may be dominant in this balance[1,2]. PAI-1 belongs to the serpin superfamily[3]. Human mature PAI-1 is a 48-kDa single-chain glycoprotein consisting of 379 amino acids[3,4]. PAI-1 is produced as an active molecule, but it spontaneously converts to an inactive, latent form[5]. Latent PAI-1 can be re-activated by exposure to denaturing agents, followed by refolding[6]. Like other serpins, PAI-1 is thought to associate with the target protease through a bait residue in a reactive site loop. The reactive site loop is partly inserted between strands 3 and 5 of the β-sheet A of the molecule, and this insertion appears to be required for inhibition[7].

In the course of identifying agents that enhance fibrinolytic activity of vascular endothelial cells, we have isolated a novel fatty acid, 11-keto-9(*E*),12(*E*)-octadecadienoic acid (KOD), from a culture of *Trichoderma* sp. F5594[8]. This report deals with the mechanism of the KOD enhancement of fibrinolytic activity of bovine aortic endothelial cells (BAEC). The results demonstrated that KOD irreversibly inactivated PAI-1 and, thereby, enhanced uPA-mediated plasminogen activation in BAEC.

Materials and Methods

Materials

Chemicals and proteins were obtained from the following sources: human plasminogen (*N*-terminal glutamic acid form) from Enzyme Research Laboratories (South Bend, IN, USA); human uPA and rabbit anti-uPA serum from JCR Pharmaceuticals (Kobe, Japan); goat anti-tPA IgG from Biopool (Umeå, Sweden); bovine fibrinogen, human thrombin, S-2251 (H-D-valyl-leucyl-lysine-*p*-nitroanilide), stearic acid, oleic acid, linoleic acid and 3-hydroxyhexadecanoic acid from Sigma (St. Louis, MO, USA); Spectrozyme UK (carbobenzoxy-L-γ-glutamyl (α-*t*-butoxy)-glycyl-arginine-*p*-nitroanilide) from American Diagnostica Inc. (Greenwich, CT, USA); [^{35}S]EXPRESS™ Protein Labeling Mix (73% L-[^{35}S]methionine, 22% L-[^{35}S]cysteine) from NEN; protein A-Sepharose from Pharmacia Biotech. PAI-1 was from two sources: one from the human melanoma cell line MJZJ (American Diagnostica) and the other from human HT1080 cells, which was purified and re-activated according to the method of LAWRENCE *et al.*[9]. KOD was isolated from cultures of *Trichoderma* sp. F5594[8]. The compositions of media and buffers were: medium A, Eagle's minimum

essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin; medium B, medium A devoid of $NaHCO_3$ but supplemented with 20 mM HEPES, pH 7.4; buffer A, 50 mM Tris-HCl and 100 mM NaCl, pH 7.4; buffer B, 150 mM NaCl and 20 mM sodium phosphate, pH 7.4; buffer C, 10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA, pH 7.5; buffer D, buffer B containing 0.05% Triton X-100; buffer E, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 100 μg/ml bovine serum albumin and 0.01% Tween 80.

Cell Culture

BAEC were isolated from bovine aorta and subcultured in medium A for approximately 6 passages. For assays, cells were seeded into 96-well tissue culture plates (5×10^4 cells/50 μl per well) or 35-mm culture dishes (1.0 to 1.5×10^6 cells/1.5 ml per dish) and grown for 24 hours before use.

Determination of Fibrinolytic Activity of BAEC

BAEC grown in 96-well plates were washed twice with medium B and preincubated at 37°C for 6 hours in 50 μl of medium B with or without KOD. At the end of the incubation, cells were washed with buffer A and then received 100 μl of buffer A containing 0.1 μM plasminogen and 0.1 mM S-2251. After incubation at 37°C for up to 4 hours, the release of *p*-nitroaniline was determined by measuring the change in absorbance at 405 nm.

Immunoprecipitation of uPA

BAEC were washed with buffer B and labeled at 37 °C for 6 hours in the presence of ^{35}S protein labeling mixture (50 μCi/ml) in methionine-free medium A. After washing with buffer B, cells were scraped in buffer C containing 10 μg/ml aprotinin and disrupted by sonication. Using the supernatant of the cell lysate (approximately 1.3×10^7 cpm), immunoprecipitation was carried out as described[10].

Reverse Fibrin Zymography

BAEC grown in 35-mm dishes were washed twice with buffer B and incubated in 750 μl of medium A at 37°C for 2 hours in the absence or presence of KOD. After washing, cells were removed by scraping in 1 ml of buffer B and centrifuged. The resulting cell pellet was dissolved in 100 μl of buffer B containing 0.5% Triton X-100. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoresis, the gel was washed twice with 250 ml of 2.5% Triton X-100 for 45 minutes to remove SDS, placed onto a fibrin-agar indicator gel[11] containing 0.2 unit/ml uPA, and incubated at 37 °C for 3~5 hours. The indicator gel was stained with 0.1% amide black in 30% methanol and 10% acetic acid and was destained in 30% methanol and 10% acetic acid.

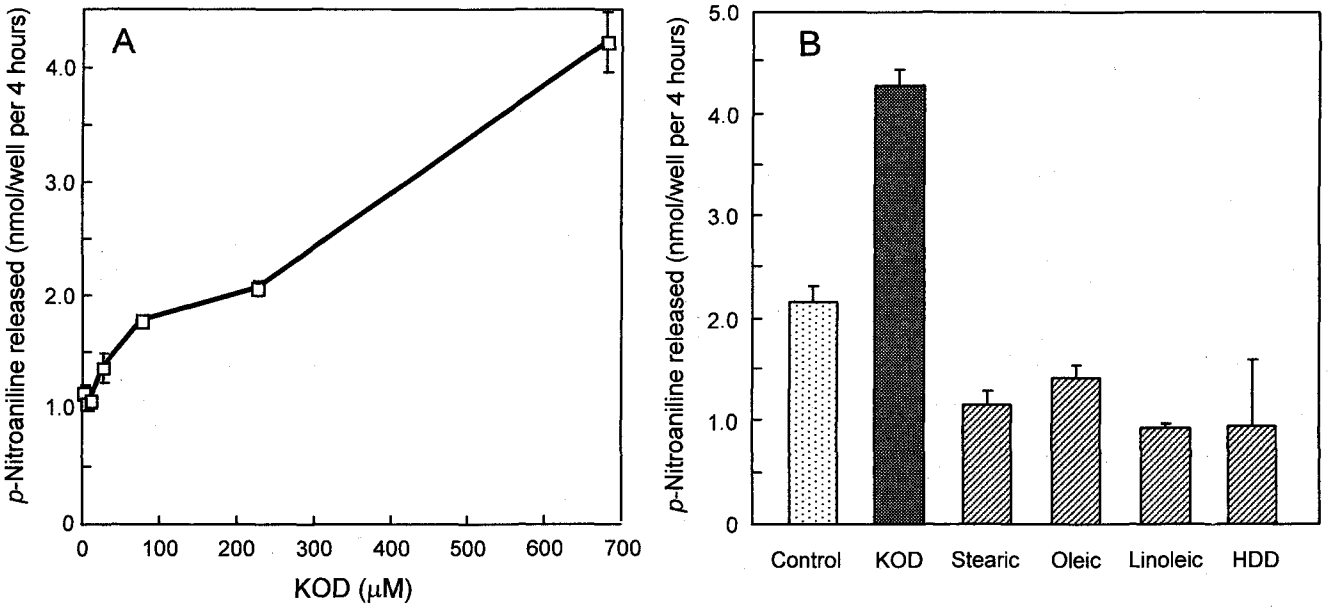
Determination of PAI-1 Activity

PAI-1 activity was determined as the inhibitory activity against uPA as follows. Active human melanoma PAI-1 (4.21 μg/ml) was preincubated with or without KOD in 50 μl of buffer D at 37°C for 15 minutes. Subsequently, 25 μl of uPA (8 units/ml) in buffer D containing 20 mg/ml bovine serum albumin were added and the mixture was incubated at 37°C for 30 minutes. Then, remaining uPA activity was determined by adding 25 μl of 0.4 mM Spectrozyme UK (in buffer D) to the mixture, followed by further incubating at 37°C to measure changes in absorbance at 405 nm. In the experiment by which reversibility of KOD inhibition was examined, re-activated PAI-1 from human HT1080 cells (2.45 μg/ml) was preincubated in buffer E at 37°C for 30 minutes in the absence or presence of KOD. The KOD-treated PAI-1 was assayed for uPA inhibition either directly or after dialysis against 4 M guanidine HCl in phosphate-buffered saline, pH 7.3 containing 0.1 mM dithiothreitol and 0.01% Tween 80 at 37°C for 4.5 hours and then against 50 mM sodium phosphate, pH 6.6, 500 mM NaCl, 0.1 mM dithiothreitol and 0.01% Tween 80 at 4°C for 16 hours[9]. For uPA inhibition assay, aliquots of the treated PAI-1 (20 μl) was incubated with 10 μl of uPA (75 units/ml in buffer E) at 37°C for 15 minutes. Subsequently, the mixture received 20 μl of Spectrozyme UK (0.25 mM in buffer E) and changes in absorbance at 405 nm were measured at 37°C.

Results

The fibrinolytic activity of BAEC was determined by incubating the cells at 37°C for 6 hours in the absence or presence of KOD, followed by washing and further incubating the cells with plasminogen and a chromogenic plasmin substrate, S-2251. KOD stimulated the activity at a concentration higher than 30 μM, and the activity doubled at ~250 μM (Fig. 1A). The elimination of plasminogen in the second incubation abolished the effect of KOD (data not shown), indicating that its effect was mediated by plasminogen activation. Stearic, oleic, linoleic and 3-hydroxyhexadecanoic acids failed to enhance, rather were inhibitory to, fibrinolytic activity at a concentration of 250~260 μM (Fig. 1B), suggesting an essential role of the

Fig. 1. Effects of KOD and other fatty acids on fibrinolytic activity of BAEC.



BAEC were preincubated at 37°C for 6 hours in the presence of the indicated concentrations of KOD (A) or the indicated fatty acids: 270 μM KOD, 250 μM stearic acid, 250 μM oleic acid, 250 μM linoleic acid or 260 μM 3-hydroxyhexadecanoic acid (HHD) (B). After washing, cells received plasminogen and S-2251 and were further incubated at 37°C for 4 hours to measure changes in absorbance at 405 nm. Each value represents the mean±S.D. from triplicate determinations.

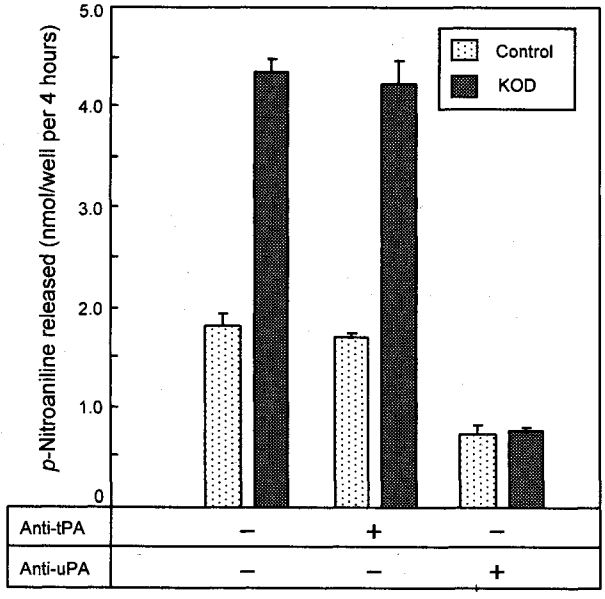
α,β-unsaturated carbonyl function in the KOD molecule.

To determine the contribution of plasminogen activators in the KOD effect, fibrinolytic activity was determined in the presence of anti-uPA and anti-tPA antibodies at concentrations inhibiting >95% of respective enzyme in a purified system. In control cells, activity was markedly reduced by anti-uPA but slightly by anti-tPA (Fig. 2), indicating that uPA was the predominant plasminogen activator in BAEC under the present experimental conditions. In KOD-treated cells, anti-tPA caused slight inhibition, while anti-uPA reduced activity to a level comparable to that in control cells. KOD failed to enhance uPA-catalyzed plasminogen activation and the resulting plasmin activity in a cell free system (data not shown); this excluded the possibility of a direct activation of uPA and/or plasmin by KOD. Thus, these results suggested that the KOD effect was mediated by a change in the amount of the active species of uPA on BAEC. However, the level of uPA in BAEC was unaffected by KOD, as revealed by immunoprecipitation of uPA that had been metabolically labeled with ^{35}S in the presence and absence of KOD. Further, the conversion of the [^{35}S]pro-uPA (single-chain form) to the two-chain form was not enhanced in cells incubated with KOD (data not shown).

To test for the possibility that the KOD effect is associated with changes in PAI activity, the level of PAI in BAEC was determined by reverse fibrin zymography after SDS-PAGE under reducing conditions, which enable the detection of PAI-1 but not PAI-2[12]. In the zymogram, cell lysate from untreated control BAEC gave a prominent lysis-resistant area that indicated the presence of PAI-1. On the other hand, lysate from KOD-treated cells produced a rather faint band, showing that PAI-1 activity in KOD-treated BAEC was significantly reduced (Fig. 3A). Furthermore, when lysate from untreated BAEC was incubated with KOD in a cell-free conditions, the intensity of a lysis-resistant band was markedly reduced as compared to that observed with lysate not incubated with KOD (Fig. 3B), indicating a direct inhibition of PAI-1 by KOD.

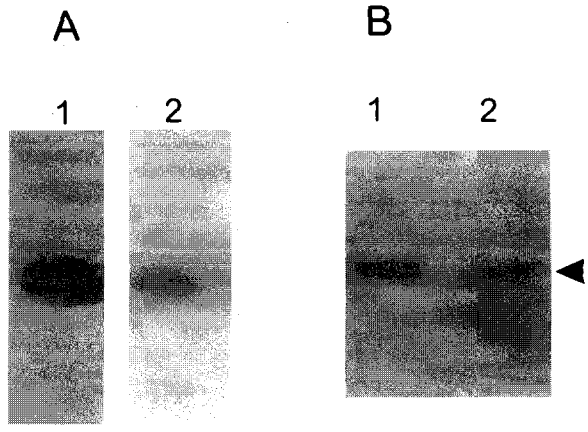
Next, the effect of KOD on human PAI-1 activity was examined in a cell-free system using purified materials (Fig. 4). In the first experiment, uPA was incubated with human PAI-1 that had been preincubated with or without KOD, following which residual uPA activity was

Fig. 2. Effects of KOD on fibrinolytic activity of BAEC in the absence and presence of anti-tPA or anti-uPA antibody.



BAEC were incubated at 37°C for 6 hours in the absence or presence of 270 μM KOD. After washing, cells were incubated at 37°C for 10 minutes with buffer A containing none, anti-tPA IgG (2.85 μg/ml) or anti-uPA serum (215 μg/ml). Subsequently each culture received plasminogen and S-2251. After further incubation at 37°C for 4 hours, changes in absorbance at 405 nm was measured. Each value represents the mean±S.D. from triplicate determinations.

Fig. 3. Effect of KOD on PAI activity in BAEC as visualized by reverse fibrin zymography.



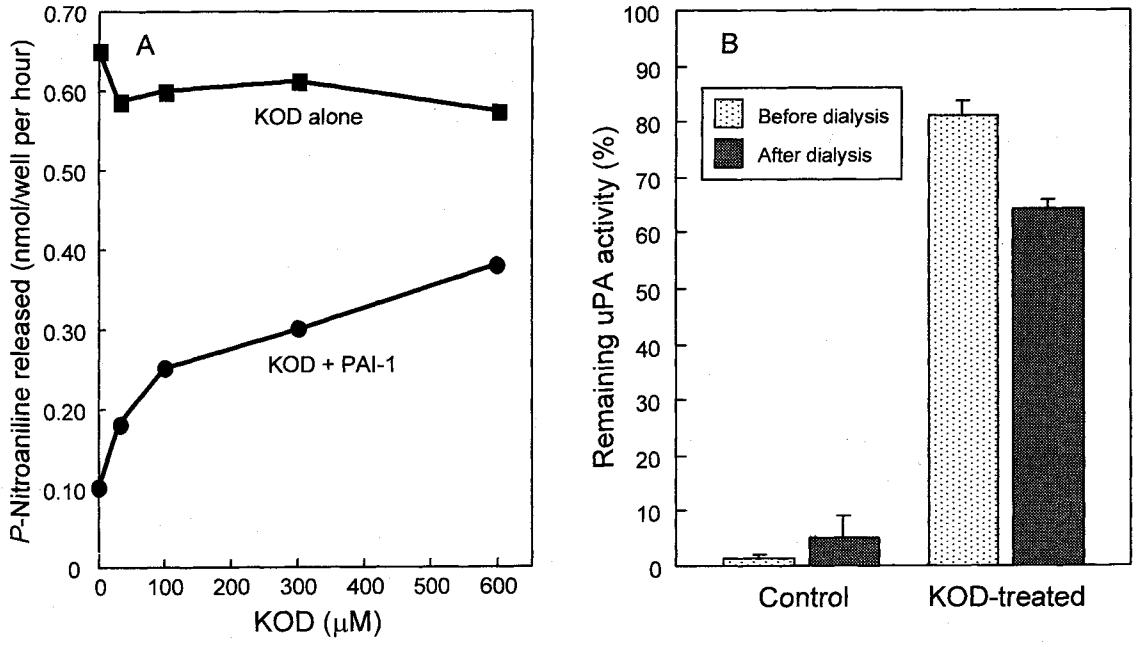
(A) BAEC were incubated at 37°C for 2 hours in the absence or presence of KOD (300 μM) in medium A. Subsequently, cell lysate (50 μg protein) was subjected to SDS-PAGE on a 10% gel under reducing conditions, and the gel was processed for reverse fibrin zymography. Lane 1, lysate from control cells; lane 2, lysate from KOD-treated cells. (B) Lysate prepared from untreated BAEC (250 μg/ml) was incubated at 37°C for 2 hours in the absence or presence of KOD (300 μM), and a portion of the mixture (5 μg protein) was subjected to SDS-PAGE on a 10% gel under reducing conditions. Subsequently, the gel was processed for reverse fibrin zymography. Lane 1, control; lane 2, KOD-treated. Arrowhead denotes the position of lysis-resistant band.

determined (Fig. 4A). The residual uPA activity was reduced to 15% by PAI-1 that had not been treated with KOD. On the other hand, KOD-treated PAI-1 became less inhibitory. The residual uPA activity was increased to 50 and 67% by PAI-1 preincubated with KOD at concentrations of 300 and 600 μM, respectively. KOD did not affect uPA activity in the absence of PAI-1. To examine whether KOD inhibition of PAI-1 involved an inactivation or of an elevated conversion of active PAI-1 to latent analogue, active PAI-1 was incubated with KOD and the treated PAI-1 was assayed for uPA inhibition before and after dialysis against guanidine HCl, which re-activates latent PAI-1. As shown in Fig. 4B, activity of KOD-treated PAI-1 to inhibit uPA was not restored even after dialysis against the denaturant; this demonstrated that an irreversible inactivation of PAI-1 occurred.

Discussion

KOD increases plasmin activity of BAEC. Although the KOD effect is not apparent in the absence of plasminogen and is blocked by anti-uPA, it enhances neither production nor activation of uPA in the cells. Further, KOD is not stimulatory to urokinase-catalyzed plasminogen activation and the resulting plasmin activity in a purified system. On the other hand, PAI-1 activities both in BAEC and in a purified system are markedly inhibited by KOD. From these results, it was concluded that KOD enhances fibrinolytic activity of BAEC, at least in part, by inhibiting PAI-1. PAI-1 is spontaneously converted to the latent form[5], which can be re-activated by exposure to denaturing agents[6]. The activity of KOD-inhibited PAI-1 is not restored by a treatment with guanidine HCl. Similarly, the KOD-treated BAEC lysate exerts much less PAI-1 activity even after electrophoresis under the denaturing conditions, which can

Fig. 4. Inhibition of PAI-1 by KOD in a purified system.



(A) The indicated concentrations of KOD were preincubated with 0 (■) or 4.21 μg/ml of human melanoma PAI-1 (●) at 37°C for 15 minutes. Subsequently, inhibition of uPA activity was determined in duplicate. (B) PAI-1 from HT1080 cells (2.45 μg/ml) was preincubated with 0 or 200 μM KOD at 37°C for 30 minutes. Aliquots of the preincubated PAI-1 were assayed for uPA inhibition either directly (before denaturation) or after denaturation by guanidine HCl. Each value represents the mean±S.D. from triplicate determinations.

restore activity of latent PAI-1. These results demonstrate that the KOD inhibition of PAI-1 is caused by an irreversible inactivation, rather than by an increased conversion into the latent form.

The α,β-unsaturated carbonyl function of the KOD molecule appears to be essential to PAI-1 inactivation. α,β-Unsaturated carbonyl compounds may cause protein modification at sulfhydryl, imidazolyl and amino groups[13,14]. The PAI-1 molecule does not contain cysteine, and histidine and lysine are not present in the essential region (P_{15} to $P_{1'}$) of the mobile reactive site loop[4], which plays a role by being partially inserted into the β-sheet A upon binding to a serine protease[5]. However, the amino acids 110~145 region, which participates in target protease binding[15], contains a histidine and three lysine residues[4]. Thus, it is suggested that KOD modification of one (or more) of these residues in the 110~145 region may be involved in PAI-1 inactivation. Recently, α,β-unsaturated ketone-containing diketopiperazines have been identified as PAI-1 inhibitors[16~18]. The observation that such compounds bind the 110~145 region[18] may support the above hypothesis.

Acknowledgments

We thank SHIGEMITSU NAKASHIMA for technical assistance and YOSHIKAZU KITANO for discussion. This work was supported in part by grants from the Ministry of Health and Welfare, Japan and the Ministry of Education, Science, Sports and Culture, Japan.

References

1) AZNAR, J. & A. ESTELLES: Role of plasminogen activator inhibitor type 1 in the pathogenesis of coronary artery diseases. Haemostasis 24: 243~251, 1994
2) WIMAN, B.: Plasminogen activator inhibitor 1 (PAI-1) in plasma: its role in thrombotic disease. Thromb. Haemost. 74: 71~76, 1995
3) VAN MEIJER, M. & H. PANNEKOEK: Structure of plasminogen activator inhibitor 1 (PAI-1) and its function in fibrinolysis: an update. Fibrinolysis 9: 263~276, 1995
4) NY, T.; M. SAWDEY, D. LAWRENCE, J. L. MILLAN & D. J. LOSKUTOFF: Cloning and sequence of a cDNA coding for the human β-migrating endothelial-cell-type plasminogen activator inhibitor. Proc. Natl. Acad. Sci. USA 83: 6776~6780, 1986
5) LINDAHL, T. L.; O. Sigurdardottir & B. Wiman: Stability of plasminogen activator inhibitor 1 (PAI-1). Thromb.

Haemost. 62: 748~751, 1989

6) HEKMAN, C. M. & D. J. LOSKUTOFF: Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. J. Biol. Chem. 260: 11581~11587, 1985

7) LAWRENCE, D. A.; S. T. OLSON, S. PALANIAPPAN & D. GINSBURG: Serpin reactive center loop mobility is required for inhibitor function but not for enzyme recognition. J. Biol. Chem. 269: 27657~27662, 1994

8) SHINOHARA, C.; K. Hasumi, T. Chikanishi, T. Kikuchi & A. Endo: 11-Keto-9(*E*),12(*E*)-octadecadienoic acid, a novel fatty acid that enhances fibrinolytic activity of endothelial cells. J. Antibiotics 52: 171~174, 1999

9) LAWRENCE, D.; L. STRANDBERG, T. GRUNDSTRÖM & T. NY: Purification of active human plasminogen activator inhibitor 1 from *Escherichia coli*. Comparison with natural and recombinant forms purified from eucaryotic cells. Eur. J. Biochem. 186: 523~533, 1989

10) STOPPELLI, M. P.; C. TACCHETTI, M. V. CUBELLIS, A. CORTI, V. J. HEARING, G. CASSANI, E. APPELLA & F. BLASI: Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell 45: 675~684, 1986

11) LOSKUTOFF, D. J. & R. SCHLEEF: Plasminogen activators and their inhibitors. Methods Enzymol. 163: 293~302, 1988

12) MISKIN, R. & R. ABRAMOVITZ: One-phase reverse zymography after denaturing gel electrophoresis: high sensitivity detection of activity of plasminogen activator inhibitor 2 and other protease inhibitors. Fibrinolysis 9: 331~342, 1995

13) UCHIDA, K.; S. TOYOKUNI, K. NISHIKAWA, S. KAWAKISHI, H. ODA, H. HIAI & E. R. STADTMAN: Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 33: 12487~12494, 1994

14) REQUENA, J. R.; M. X. FU, M. U. AHMED, A. J. JENKINS, T.J. LYONS, J.W. BAYNES & S.R. THORPE: Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. Biochem. J. 322: 317~325, 1997

15) KEIJER, B. J.; M. LINDERS, A.-J. VAN ZONNEVELD, H. J. EHRLICH, J.-P. DE BOER & H. PANNEKOEK: The interaction of plasminogen activator inhibitor 1 with plasminogen activators (tissue-type and urokinase-type) and fibrin: localization of interaction sites and physiologic relevance. Blood 78: 401~409, 1991

16) BRYANS, J.; P. CHARLTON, I. CHICARELLI-ROBINSON, M. COLLINS, R. FAINT, C. LATHAM, I. SHAW & S. TREW: Inhibition of plasminogen activator inhibitor-1 activity by two diketopiperazines, XR330 and XR334 produced by *Streptomyces* sp. J. Antibiotics 49: 1014~1021, 1996

17) CHARLTON, P. A.; R. W. FAINT, F. BENT, J. BRYANS, I. CHICARELLI-ROBINSON, I. MACKIE, S. MACHIN & P. BEVAN: Evaluation of a low molecular weight modulator of human plasminogen activator inhibitor-1 activity. Thromb. Haemost. 75: 808~815, 1996

18) FRIEDERICH, P. W.; M. LEVI, B. J. BIEMOND, P. CHARLTON, D. TEMPLETON, A. J. VAN ZONNEVELD, P. BEVAN, H. PANNEKOEK & J. W. TEN KATE: Novel low-molecular-weight inhibitor of PAI-1 (XR5118) promotes endogenous fibrinolysis and produces postthrombolysis thrombus growth in rabbits. Circulation 96: 916~921, 1997