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Enhancement of Fibrinolytic Activity of Vascular Endothelial Cells by Chaetoglobosin A, Crinipellin B, Geodin and Triticone B

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Four fungal metabolites, chaetoglobosin A (CGA), crinipellin B (CPB), geodin (GE) and triticone B (TTB), were found to enhance fibrinolytic activity of bovine aortic endothelial cells. Plasmin generation on the cells was elevated 2- to 4-fold when treated with these agents at a concentration of 3~100 μM. These effects were dependent on plasminogen and inhibited by anti-urokinase antibody. The effect of CGA, but not of CPB, GE and TTB, was abolished by cycloheximide. In a cell-free system, plasmin and urokinase activities as well as urokinase-catalyzed plasminogen activation were not enhanced by these agents. CGA, but not others, induced the production of urokinase in endothelial cells. CPB and GE accelerated plasminogen activator inhibitor-1 (PAI-1) inactivation, and TTB caused direct, reversible inhibition of PAI-1. Thus, induction of urokinase by CGA and inhibition of PAI-1 by CPB, GE and TTB may, at least partly, account for the elevation of fibrinolytic activity of endothelial cells.

The plasminogen/plasmin system is involved not only in blood clot dissolution but also in a variety of physiological and pathological processes requiring localized proteolysis[1,2]. In this system, the zymogen plasminogen is proteolytically activated by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), which are regulated by their inhibitors. Vascular endothelial cells synthesize and secrete plasminogen activators as well as plasminogen activator inhibitors. The balance of production between plasminogen activators and their inhibitors by endothelial cells mainly regulates fibrinolysis in the blood vessel[3]. In patients with atherosclerosis and thromboembolism, the inhibitor may be dominant in this balance. Indeed, it has been reported that elevated plasminogen activator inhibitor-1 (PAI-1) level is a risk factor in thrombotic disease and that PAI-1 expression is increased in atherosclerotic arteries[4~8].

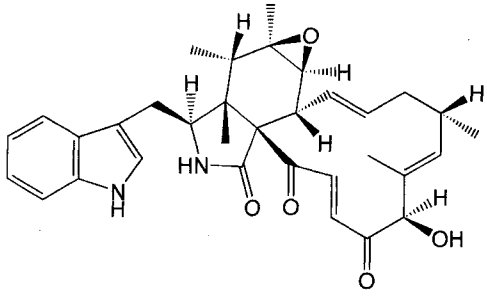
The present investigation was undertaken to identify agents that can enhance fibrinolytic activity of vascular endothelial cells. We have screened microorganisms, including actinomycetes and fungi, for their ability to produce stimulatory compounds. As a result, four fungal metabolites, chaetoglobosin A (CGA), crinipellin B (CPB), geodin (GE) and triticone B (TTB), have been identified as active compounds (Fig. 1). In this paper, we describe biochemical characterization of the effects of these agents.

Materials and Methods

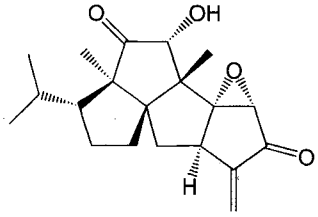
Materials

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-human uPA serum from JCR Pharmaceuticals (Kobe, Japan); goat anti-human tPA IgG from Biopool (Umeå, Sweden); bovine serum albumin, human plasmin, methionine-free EAGLE's minimum essential medium, aprotinin and S-2251 (H-D-valyl-leucyl-lysine-*p*-nitroanilide) 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^{TM} Protein Labeling Mix (73% L-[^{35}S]methionine, 22% L-[^{35}S]cysteine) from NEN; protein A-Sepharose from Pharmacia Biotech. PAI-1 was purified according to the method of LAWRENCE *et al.*[9] from conditioned medium of human HT1080 cells. The compositions of media and

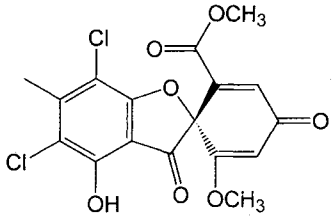
Fig. 1. Structures of chaetoglobosin A (CGA), crinipellin B (CPB), geodin (GE) and triticone B (TTB).



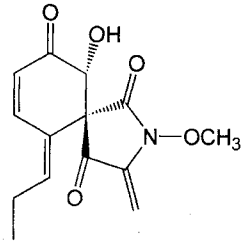
Chaetoglobosin A (CGA)



Crinipellin B (CPB)



Geodin (GE)



Triticone B (TTB)

buffers were as follows: 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 containing 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, 150 mM NaCl, 50 mM Tris-HCl, 100 μg/ml bovine serum albumin and 0.01% Tween 80, pH 7.5.

Isolation of CGA, CPB, GE and TTB

CGA, CPB, GE and TTB were isolated from cultures of *Ascotricha chartarum* IFO 6310, *Crinipellis stipitaria* IFO 30259, an unidentified fungus F6046, and *Preussia terricola* IFO 7896, respectively, by a combination of solvent extraction and column chromatography. The structure of each compound was determined by spectroscopic analyses including mass, NMR, UV and IR as well as X-ray crystallography.

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta and subcultured in medium A for 6~10 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 Plasmin Activity of BAEC

BAEC grown in 96-well plates were washed twice with medium B and preincubated at 37°C for varying time in 50 μl of medium B with or without test samples. At the end of the incubation, cells were washed with ice-cold 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 6 hours, the release of *p*-nitroaniline (*p*NA) was determined by measuring the change in absorbance at 405 nm using a model 450 microplate reader (Bio-Rad).

Cell-free Assay for Plasmin, uPA and Plasminogen Activation by uPA

Plasmin and uPA activities were determined in 100 μl of buffer A containing 0.01% Tween 80, using S-2251 or Spectrozyme UK, respectively, as a substrate. The release of *p*NA was measured at 37°C as described above. The enzyme and substrate concentrations were: 5 nM and

0.1 mM for plasmin; 5 units/ml and 0.1 mM for uPA. In the assay for plasminogen activation by uPA, 0.1 μM plasminogen and 0.1 mM S-2251 were incubated at 37°C with uPA (0.5 unit/ml) in 100 μl of buffer A containing 0.01% Tween 80. From the slope of the plots of A_{405} nm versus t^2, initial velocity of plasmin generation was calculated.

Immunoprecipitation of uPA

BAEC grown in 35-mm dishes were washed with buffer B and were labeled at 37°C for 6 or 18 hours in the presence of test sample and ^{35}S protein labeling mixture (50 μCi/ml) in methionine-free medium A. When the conversion of two-chain uPA to single-chain uPA was to be determined, BAEC labeled for 6 hours in the absence of test sample were washed twice and chased for 2 hours at 37°C with test sample in methionine-containing medium A. After washing with buffer B, cells were scraped in 0.5 ml of buffer C and dissolved by sonication in an ice bath. Aprotinin (10 μg/ml) was added to the lysate, and the mixture was centrifuged at 10,000×g for 10 minutes at 4°C to obtain supernatant. Protein A-Sepharose (10 μl) was added to the supernatant (1.3×10^7 cpm of trichloroacetic acid-insoluble radioactivity in 0.5 ml buffer C), and the mixture was incubated for 1 hour at 4°C. After centrifugation at 10,000×g for 2 minutes, the resulting supernatant (450 μl) was incubated with rabbit anti-uPA serum (35.8 μg/ml) for 30 minutes at 22°C, then with 10 μl of protein A-Sepharose at 4°C for 1 hour. The mixture was centrifuged, and the resulting pellet was washed five times with 1 ml of buffer C and three times with 1 ml of 40 mM HEPES, pH 7.5. The immunoprecipitates were dissolved in 20 μl of LAEMMLI's sample buffer under reducing conditions at 37°C for 30 minutes and subjected to SDS-polyacrylamide gel electrophoresis (PAGE)[10]. After electrophoresis, gels were treated with Amplify (Amersham) and processed for fluorography. The gel was exposed to an X-ray film for one week at −80°C.

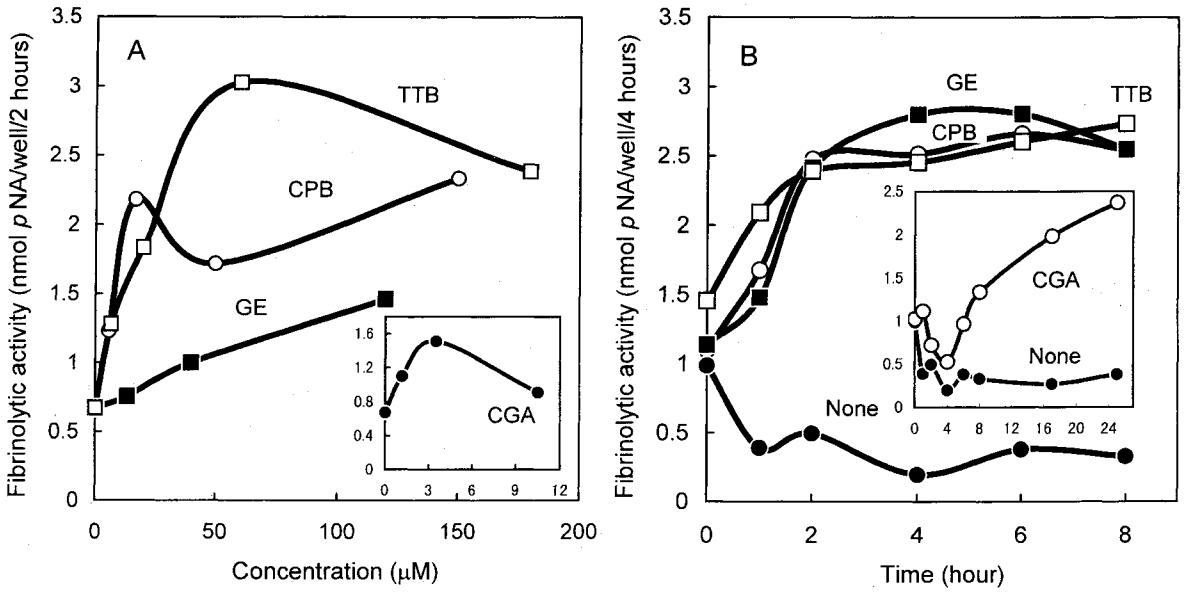
Determination of PAI-1 Activity

PAI-1 activity was determined as the inhibitory activity toward uPA as follows. Purified human PAI-1 (2.45 μg/ml) was preincubated with or without test sample in 20 μl of buffer D at 37°C for varying time. The mixture received 10 μl of uPA (75 units/ml in buffer D) and was incubated at 37°C for 10 minutes. Subsequently, 20 μl of Spectrozyme UK (0.25 mM in buffer D) were added the mixture, and changes in absorbance at 405 nm were measured at 37°C for up to 60 minutes. In experiments by which reversibility of PAI-1 inhibition was examined, PAI-1 (2.45 μg/ml) was preincubated in buffer D at 37°C for 5 hours in the absence or presence of test sample. The treated PAI-1 was assayed for uPA inhibition either directly or after dialysis against 4 M guanidine hydrochloride 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 (20 μl) of the treated PAI-1 was incubated with uPA, and remaining uPA activity was assayed as described above.

Results

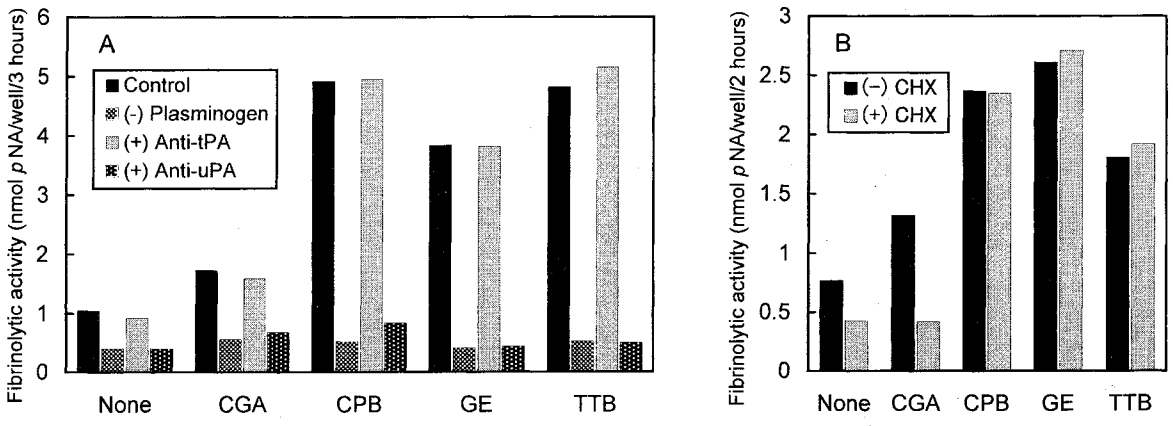
The fibrinolytic activity of BAEC was determined as plasmin activity on the cells. In this determination, cells were incubated at 37°C for 6 hours in the presence of test samples, washed and re-incubated with plasminogen and a chromogenic plasmin substrate, S-2251. A screening of microbial cultures for their ability to enhance this activity resulted in the identification of four active compounds, CGA, CPB, GE, and TTB (Fig. 1). These agents enhanced the activity at concentrations ranging from 1~100 μM: the concentration required for 2-fold elevation was approximately 3 μM for CGA, 8~15 μM for both CPB and TTB, and 90 μM for GE (Fig. 2A). The time course of the effects of these agents is shown in Fig. 2B. CPB, GE and TTB exerted their maximal activity after 2~8 hours of treatment, while the effect of CGA appeard after 6 hours and became prominent after 18~25 hours. The elimination of plasminogen in the second incubation resulted in no increase in fibrinolytic activity in cells treated with these agents (Fig. 3A), indicating that these effects were mediated by plasminogen activation. The addition of anti-uPA but not anti-tPA antibody in the second incubation caused marked reduction in activity not only in control cells but also in cells treated with these agents (Fig. 3A). Thus, these results demonstrated that the effects of these agents were mediated by uPA-catalyzed activation of plasminogen. However, in a cell-free purified system, these agents enhanced activity of neither plasmin, uPA, nor uPA-catalyzed activation of plasminogen. Respective enzyme activities (% of control) in the presence of compounds were: 103±1, 101±1 and 98±7 for CGA; 98±1, 99±2 and 96±2 for CPB; 97±1, 100±0 and 95±12 for GE; 96±2, 100±2 and 101±11 for TTB. These results exclude the possibility that these agents cause a direct activation of uPA and/or plasmin. Cycloheximide completely abolished

Fig. 2. Enhancement of plasmin activity of BAEC by CGA, CPB, GE and TTB.



BAEC were preincubated at 37°C for 6 hours (A) or the indicated time (B) in the presence of CGA, CPB, GE and TTB at the indicated concentrations. The concentrations used in panel B were 3 μM for CGA, 15 μM for CPB, 100 μM for GE and 20 μM for TTB. After washing, cells received plasminogen and S-2251 and were further incubated at 37°C for 2 (A) or 4 hours (B) to determine cellular plasmin activity. Each value represents the average of duplicate determinations.

Fig. 3. Effects of plasminogen, anti-plasminogen activator antibodies and cycloheximide on plasmin activity of BAEC treated with CGA, CPB, GE and TTB.



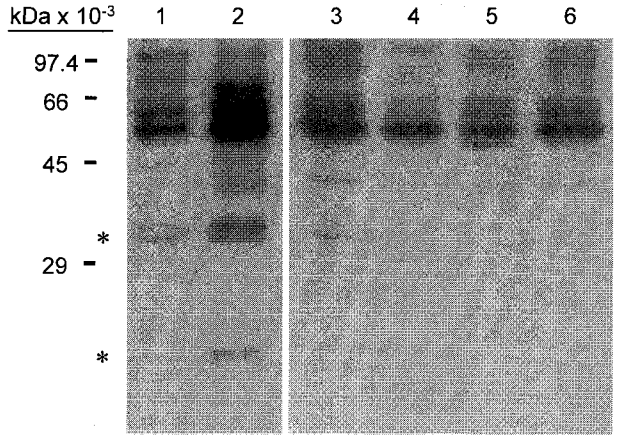
(A) BAEC were incubated at 37°C for 6 hours in the absence or presence of CGA (3 μM), CPB (15 μM), GE (100 μM) or TTB (20 μM). After washing, cells were incubated at 37°C for 10 minutes with buffer A containing none, anti-tPA IgG (14.1 μg/ml) or anti-uPA serum (110 μg/ml). Subsequently each culture received S-2251 alone or S-2251 and plasminogen. After further incubation at 37°C for 3 hours, changes in absorbance at 405 nm was measured. (B) BAEC were preincubated with or without CGA (3 μM), CPB (15 μM), GE (100 μM) or TTB (20 μM) at 37°C for 6 hours in the absence or presence of 10 μg/ml cycloheximide (CHX). After washing, cells received plasminogen and S-2251 and were further incubated at 37°C for 2 hours to determine fibrinolytic activity. Each value represents the average of triplicate determinations.

the CGA effect, while the effects of CPB, GE and TTB were not affected by the protein synthesis inhibitor (Fig. 3B).

uPA synthesis in BAEC was determined by labeling cells with ^{35}S, followed by immunoprecipitation and SDS-PAGE analysis of [^{35}S]uPA. In cells labeled for 18 hours in the presence of CGA, the level of [^{35}S]single-chain uPA as well as [^{35}S]two-chain uPA was markedly elevated as compared with control cells (Fig. 4). Consistently, fibrin zymography of extracts of CGA-treated cells demonstrated an increase in the amount of uPA (data not shown). The treatment with CPB, GE and TTB did not enhance uPA production (Fig. 4). In addition, the rates of the conversion of [^{35}S]single-chain uPA to the two-chain form, as determined by chasing ^{35}S-labeled cells for 2 hours in the presence of CPB, GE and TTB, respectively, were 14.5%, 9.6% and 12.9%, which was comparable to that in non-treated cells (13.3%). These results demonstrated that CGA induced the synthesis of uPA in BAEC and that the effects of CPB, GE and TTB were related neither to uPA synthesis nor conversion of single-chain uPA to active two-chain form.

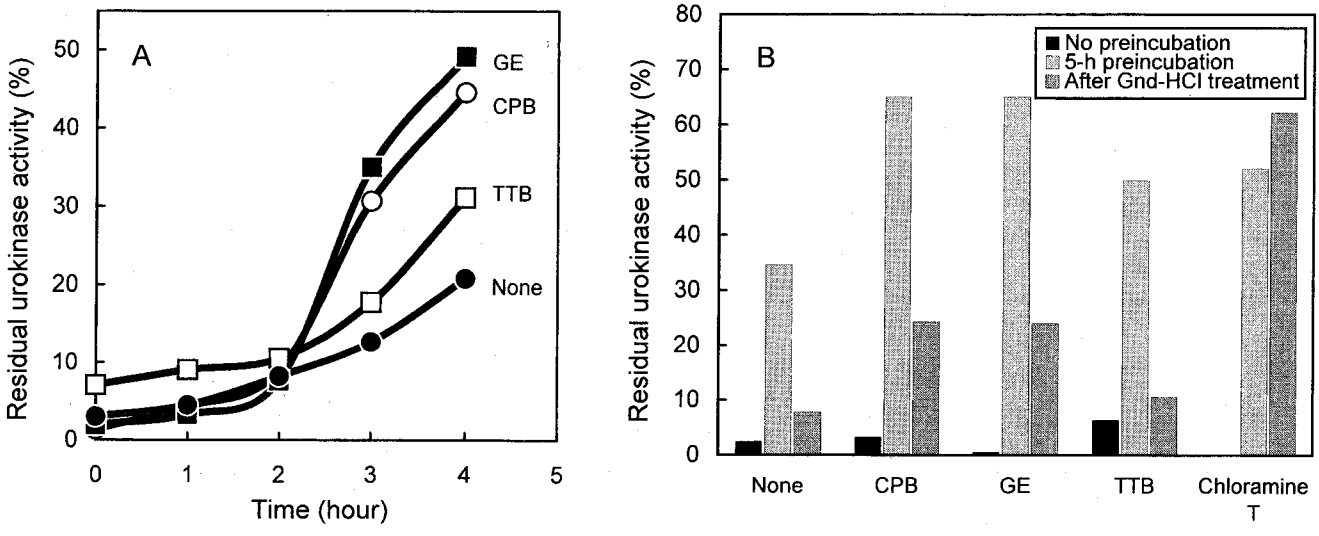
BAEC produce PAI-1[11], which regulates fibrinolytic activity by inhibiting plasminogen activators. Effects of CPB, GE and TTB on PAI-1 activity were investigated using purified, re-activated human PAI-1. In the first

Fig. 4. Immunoprecipitation of [^{35}S]uPA from BAEC treated with CGA, CPB, GE and TTB.



BAEC were incubated with or without CGA (3 μM), CPB (15 μM), GE (100 μM) or TTB (20 μM) at 37°C for 6 hours (lanes 3~6) or 18 hours (lanes 1 and 2) in a methionine-free medium containing ^{35}S protein labeling mixture. After incubations, cell lysates were prepared and subjected to immunoprecipitation with rabbit anti-uPA serum. Lanes 1 and 3, control; lane 2, CGA; lane 4, CPB; lane 5, GE; lane 6, TTB. Positions of molecular weight standards as well as the heavy and light chains of two-chain uPA (asterisks) are shown.

Fig. 5. Inhibition of PAI-1 by CPB, GE and TTB.



(A) Time course. Human PAI-1 (2.45 μg/ml) was preincubated in the absence or presence of 150 μM of CPB, GE or TTB at 37°C for the indicated time. Subsequently, the mixture was incubated with uPA and assayed for residual uPA activity. (B) Reversibility of PAI-1 inhibition. PAI-1 (2.45 μg/ml) was preincubated with CPB (100 μM), GE (100 μM), TTB (100 μM) or chloramine T (50 μM) at 37°C for 0 or 5 hours. Subsequently, aliquots of the treated PAI-1 were assayed for uPA inhibition either directly (5-hour preincubation) or after denaturation by guanidine HCl (after Gnd-HCl treatment). Each value represents the average of duplicate determinations.

experiment, PAI-1 was preincubated with or without the agents, following which activity of the treated PAI-1 to inhibit titrating amount of uPA was determined. In control incubations, PAI-1 activity decreased (remaining uPA activity increased) as the preincubation time prolonged (Fig. 5A). This observation was consistent with the spontaneous conversion to the latent form[12]. In the presence of CPB and GE, the rate of inactivation was accelerated after 2~4 hours of preincubation, while direct inhibition of PAI-1 (time 0 of preincubation) by CPB and GE was not seen (Fig. 5A). On the other hand, TTB inhibited PAI-1 activity without preincubation and affected PAI-1 inactivation only slightly. Next, activity of drug-treated PAI-1 was determined before and after dialysis against guanidine HCl to examine whether the apparent reduction in PAI-1 activity was caused by true inactivation or by an increased conversion to the latent form. As shown in Fig. 5B, activities of CPB- and GE-treated PAI-1 to inhibit uPA were partly restored by the denaturant treatment, while TTB-treated PAI-1 recovered full activity. The partial reversibility of CPB and GE effects was evident when compared with the action of chloramine T[13], which oxidizes the P_1' Met of PAI-1 and causes irreversible inactivation (Fig. 5B).

Discussion

In the present study, four fungal metabolites, CGA, CPB, GE and TTB, have been found to enhance plasmin activity of BAEC. Although these effects appear to be dependent on plasminogen activation by urokinase, these compounds do not enhance plasmin and uPA activities as well as uPA-catalyzed plasminogen activation in a purified system. CGA induces uPA synthesis in BAEC, while CPB, GE and TTB affect neither uPA synthesis nor pro-uPA conversion to the active two-chain form. TTB inhibits PAI-1 directly, and CPB and GE accelerate PAI-1 inactivation. However, as compared with the concentrations required to enhance fibrinolytic activity of BAEC, the concentrations of these agents needed to inhibit PAI-1 are rather high. This discrepancy may be due to the higher concentration of PAI-1 used in the cell-free system and/or to structural difference between human PAI-1 and the bovine counterpart. It is suggested that the induction of uPA by CGA and the inhibition of PAI-1 by CPB, GE and TTB may, at least partly, account for the fibrinolytic activation in BAEC.

It is intriguing to note the difference between PAI-1 inhibition by TTB and that by CPB and GE. The TTB inhibition is direct and reversed by denaturation and following renaturation of PAI-1, while the PAI-1 inactivation that is accelerated by CPB and GE is only partly reversed by such treatments. The PAI-1 inhibition by these agents is also different from that by 11-keto-9(*E*),12(*E*)-octadecadienoic acid, which irreversibly inactivates PAI-1[14].

Biological properties of CPB and GE have first been described as antibiotics[15,16]. TTB has been isolated as phytotoxins[17]. CGA is an inhibitor of microfilament polymerization[18]. Effects of these agents on the fibrinolytic system have not been reported. However, it has recently reported that cytochalasin, another inhibitor of microfilament polymerization, disrupts cytoskeletal reorganization, which is implicated in the expression of AP-1-regulated genes, including uPA[19]. It is likely that CGA induces uPA by employing a similar mechanism in BAEC.

Acknowledgments

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