

Stachybotrydial Selectively Enhances Fibrin Binding and Activation of Glu-plasminogen

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Abstract Stachybotrydial, a triprenyl phenol metabolite from a fungus, has a plasminogen modulator activity selective to Glu-plasminogen. Stachybotrydial enhanced fibrin binding and activation of Glu-plasminogen (2- to 4-fold enhancement at 60~120 μ M) but not of Lys-plasminogen. Approximately 1.2~1.6 moles of [3 H]stachybotrydial bound to Glu-plasminogen to exert such effects. The selective modulation of the Glu-plasminogen function by stachybotrydial may be related to alteration of its conformational status.

Keywords nonlysine-analog plasminogen modulator, plasminogen activation, conformation, fibrinolysis, stachybotrydial

Introduction

The plasminogen/plasmin system is involved not only in fibrinolysis but also in many other physiological and pathological processes requiring localized proteolysis, including inflammation, tissue remodeling, ovulation, tumor metastasis and tissue invasion of pathogens [1~5]. In this system, the zymogen plasminogen is proteolytically activated into plasmin by urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). Circulating plasminogen with an *N*-terminal Glu (Glu-plasminogen) is a single-chain glycoprotein with multiple functional domains, which consist of an *N*-terminal peptide, five homologous kringle domains and a trypsin-

like serine protease domain [6, 7]. The binding of plasminogen to fibrin and cell surfaces localizes fibrinolytic activity on these surfaces [8]. The plasminogen binding is mediated by lysine binding sites located within the kringle domains [9, 10]. Glu-plasminogen exhibits a tight, spiral structure [11] due to an intramolecular interaction between a lysine residue(s) (Lys⁵⁰ and/or Lys⁶²) in the *N*-terminal peptide and the lysine-binding site in kringle 5 domain [12~15]. The tight conformation of Glu-plasminogen attenuates both its activation and binding to fibrin and cellular receptors [16~18]. Lysine analogs, such as 6-aminohexanoic acid, bind to the lysine binding sites and induces a large-scale conformational change in Glu-plasminogen [13, 19, 20], facilitating its activation to plasmin. Conformational regulation of Glu-plasminogen activation is also revealed by using several monoclonal antibodies that bind to kringle domains [21]. Hydrolysis of Glu-plasminogen by plasmin yields a truncated form of plasminogen called Lys-plasminogen, which predominantly has Lys⁷⁸ as an *N*-terminal residue [22, 23]. Lys-plasminogen adopts a relaxed, extended conformation, and such conformation is implicated in an increased susceptibility to activation and a high affinity fibrin binding of Lys-plasminogen [13, 16, 24, 25]. Thus, conformational status of plasminogen is implicated in localized activation of plasminogen.

We recently discovered several nonlysine-analog plasminogen modulators such as staplabin/SMTPs and thioplabin [26~34]. These low molecular mass compounds induce conformational change in plasminogen and enhance both plasminogen binding to fibrin and activation of plasminogen, leading to increased fibrinolysis. With respect to induction of conformational change in plasminogen, nonlysine-analogs are similar to lysine analogs. The striking difference between the two types of plasminogen

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modulators is that nonlysine-analogs enhance plasminogen-fibrin binding and fibrinolysis, while lysine analogs inhibit these activities. In this study, we have found that stachybotrydial [35], a triprenyl phenol metabolite from the fungus *Stachybotrys* sp., has distinct properties as compared with the previously identified nonlysine-analog plasminogen modulators. Stachybotrydial selectively enhances fibrin binding and activation of Glu-plasminogen, without increasing such activities of Lys-plasminogen.

Materials and Methods

Materials

Proteins and chemicals were from the following sources: human Glu-plasminogen and Lys-plasminogen from Enzyme Research Laboratories (South Bend, IN, USA); two-chain uPA from JCR Pharmaceuticals (Kobe, Japan); two-chain tPA from American Diagnostica (Greenwich, CT, USA); aprotinin from CosmoBio (Tokyo, Japan); 6-aminohexanoic acid, fibrinogen, thrombin, plasmin and S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide) from Sigma (St. Louis, MO, USA); Flavigen tPA (CH₃SO₂-D-HHT-Gly-Arg-*p*-nitroanilide) from Biopool (Umeå, Sweden); glutaryl-Gly-Arg-7-amino-4-methylcoumarin (Glt-Gly-Arg-AMC) from Cambridge Research Biochemicals (Cheshire, UK); Na¹²⁵I from Amersham Biosciences (Piscataway, NJ, USA); sodium [³H]acetate (methyl-³H) from Moravac Biochemicals (Brea, CA, USA).

Stachybotrydial and its congeners F1839-A, -B, -C, and -E were isolated from cultures of *Stachybotrys* sp. F1839 as described previously [35]. Radioiodination of Glu-plasminogen and Lys-plasminogen was performed using the chloramine-T method [30]. The compositions of buffers were: buffer A, 20 mM sodium phosphate and 150 mM NaCl, pH 7.4; buffer B, Hanks' balanced salt solution containing 1 mg/ml bovine serum albumin and 50 mM Hepes, pH 7.4; buffer C, 50 mM Tris-HCl, 100 mM NaCl and 0.01% (wt/vol) Tween 80, pH 7.4; buffer D, bovine serum albumin-free buffer B; buffer E, buffer A containing 2.5 mg/ml gelatin and 0.01% Tween 80.

Production and Isolation of [³H]stachybotrydial

Stachybotrys sp. F1839 [35] was grown aerobically at 28°C in 15 ml of medium consisting of 30 mg/ml glucose, 10 mg/ml soybean meal, 3 mg/ml meat extract, 3 mg/ml peptone, 3 mg/ml yeast extract, 0.5 mg/ml KH₂PO₄, and 0.5 mg/ml MgSO₄. After 91 hours, sodium [³H]acetate (370 MBq) was added to the medium, and the culture was further incubated for 29 hours. The resulting culture was extracted three times with 1 volume of 2-butanone. After

concentration, the organic extracts were applied to a silica gel column (Wakogel C-200; 10×150 mm). The column was developed successively with 30 ml of *n*-hexane, 60 ml of *n*-hexane-ethyl acetate (4:1) and 60 ml of *n*-hexane-ethyl acetate (3:2). [³H]Stachybotrydial was found in the 3:2 fraction, which was concentrated and subjected to HPLC on an Inertsil PREP-Sil column (GL Sciences, Tokyo, Japan; 6×250 mm) developed with *n*-hexane-ethyl acetate (7:3), yielding 84 μg of purified [³H]stachybotrydial. The specific radioactivity of the preparation was 117 Bq/nmol.

Determination of [¹²⁵I]plasminogen Binding to Fibrin

Human fibrinogen (0.2 mg/ml) in 100 μl buffer A was dried in 96-well tissue culture plates by incubating at 37°C for 3~5 days. Each well received 75 μl of human thrombin (0.68 international unit/ml in buffer A) and was incubated at 37°C for 3 hours to form fibrin clots. The resulting fibrin clots were washed 3 times with 100 μl of buffer A and incubated at 37°C for 60 minutes with 50 μl of buffer B containing 50 nM [¹²⁵I]plasminogen in the presence or absence of 20 mM 6-aminohexanoic acid. After washing with buffer A (twice with 200 μl and once with 100 μl), the bound [¹²⁵I]plasminogen was dissolved in 50 μl of 0.2 M NaOH and 2% (wt/vol) SDS at 37°C for 30 minutes. An aliquot (40 μl) of the lysate was counted for radioactivity. The specific binding of [¹²⁵I]plasminogen was calculated by subtracting values obtained in the presence of 20 mM 6-aminohexanoic acid (nonspecific binding) from values obtained in its absence (total binding).

Determination of Plasminogen Activation

The activation of plasminogen into plasmin was assayed in two ways: measurement of initial velocity for plasmin generation using a chromogenic substrate for plasmin (S-2251) and determination of the conversion of [¹²⁵I]-plasminogen to [¹²⁵I]plasmin using SDS-polyacrylamide gel electrophoresis. In the former assay, 0.1 μM plasminogen and 0.1 mM S-2251 were incubated in buffer C with tPA (20 units/ml) at 37°C for up to 120 minutes with monitoring the change in absorbance at 405 nm. From the slope of the plots of A₄₀₅ nm versus t² [36], initial velocity of plasmin generation was calculated. In the latter assay, 0.1 μM [¹²⁵I]plasminogen was incubated with tPA (600 units/ml) and aprotinin (1000 kallikrein inhibitor units/ml) in 15 μl of buffer C. After incubation at 37°C for 120 minutes, the mixture received 4.5 mg of solid urea and 5 μl of 3.6% (wt/vol) SDS, 3.6% (wt/vol) 2-mercaptoethanol, and 0.08% (wt/vol) bromophenol blue. After heating at 60°C for 30 minutes, 10 μl of the mixture was subjected to SDS-polyacrylamide gel electrophoresis

on a 10% gel, followed by autoradiography.

Determination of the Activities of Plasmin, tPA and uPA

The activities of plasmin, tPA and uPA were determined at 37°C in 50~80 μ l of buffer C using 100 μ M of S-2251, Flavigen tPA and Glt-Gly-Arg-AMC, respectively, as chromogenic substrates. The enzyme concentrations were: 20 nM for plasmin; 20 units/ml for tPA; 5 units/ml for uPA. The release of *p*-nitroaniline (for S-2251 and Flavigen tPA) or 7-amino-4-methylcoumarin (for Glt-Gly-Arg-AMC) was measured as a change in absorbance at 405 nm or fluorescence (excitation at 380 nm and emission at 480 nm), respectively.

Determination of [¹²⁵I]Fibrin Degradation

[¹²⁵I]Fibrin-coated well (20 μ g of [¹²⁵I]fibrin; $\sim 4 \times 10^4$ cpm) prepared as described previously [37] was incubated at 37°C for 60 minutes with 70 μ l of buffer E containing 0.1~1 international unit/ml uPA and 100 nM plasminogen. After incubation, radioactivity released from the [¹²⁵I]fibrin clot was determined. Value obtained in the absence of plasminogen was used as a blank.

[³H]Stachybotrydial Binding to Plasminogen

Plasminogen (3.0 μ M) and [³H]stachybotrydial (120 μ M) were incubated at 37°C for varying times in 15 μ l of buffer C. After incubation, the mixture received 450 μ l of acetone and was stored at -80°C for 1 hour. The mixture was centrifuged at 9,000 $\times g$ for 5 minutes at 4°C to collect plasminogen as an insoluble pellet, which was further washed twice with acetone (450 μ l). The resulting pellet was dissolved in 50 μ l of 0.2 M NaOH and an aliquot (40 μ l) was counted for radioactivity. With this acetone wash procedure, plasminogen protein was recovered quantitatively, and >99.95% of unbound [³H]stachybotrydial was removed.

Results

Stachybotrydial enhanced the binding of [¹²⁵I]Glu-plasminogen to fibrin by 1.6- to 2.7-fold at concentrations of 50~200 μ M (Fig. 1A). On the other hand, fibrin binding of [¹²⁵I]Lys-plasminogen, a conformationally relaxed plasminogen species, was not elevated by stachybotrydial (Fig. 1B). Four stachybotrydial analogs (F1839A, B, C and E) were inactive at 200~400 μ M in enhancing Glu-plasminogen binding (Fig. 1A). The structural difference between stachybotrydial and the analogs is that the analogs have a lactam moiety instead of an aromatic dialdehyde structure.

To explore the mechanism of the stachybotrydial effect, we first treated [¹²⁵I]Glu-plasminogen with 120 μ M stachybotrydial and then assayed for fibrin binding after dilution to reduce stachybotrydial concentration. The binding of the stachybotrydial-pretreated [¹²⁵I]Glu-plasminogen increased depending on the time of pretreatment (Fig. 2A). The stachybotrydial pretreatment at concentrations of 60~200 μ M caused a dose-dependent increase in [¹²⁵I]Glu-plasminogen binding (Fig. 2B). These results suggested that an interaction between stachybotrydial and Glu-plasminogen caused increases in fibrin binding. On the other hand, the fibrin binding of stachybotrydial-treated [¹²⁵I]Lys-plasminogen was not increased prominently (Fig. 2B).

The previously isolated plasminogen modulators (such as staplabin/SMTPs and thioplabins) enhance both fibrin binding and activation of plasminogen [27~29, 32]. Similarly, stachybotrydial elevated tPA-catalyzed activation of plasminogen. The stachybotrydial treatment resulted in time- and dose-dependent increases in the activation of Glu-plasminogen (Fig. 3A and B), whereas the activation of Lys-plasminogen was inhibited (Fig. 3B). Similar results were obtained when uPA was used as a plasminogen activator (data not shown). The enhancement of the activation of Glu-plasminogen accompanied an increase in the conversion of Glu-plasminogen to plasmin as shown in Fig. 3C [plasmin consists of two polypeptide chains (A- and B-chains) that are held together by disulfide bonds, and the intensity of the two chains was increased by stachybotrydial]. Stachybotrydial did not enhance the activity of plasmin, tPA and uPA when assayed using synthetic chromogenic substrates (Fig. 3D). Thus, the stachybotrydial effect did not involve direct activation of these enzymes, and it seemed likely that the effect was mediated by the interaction with Glu-plasminogen.

Stachybotrydial enhanced the degradation of fibrin clots. When [¹²⁵I]fibrin was incubated with stachybotrydial-treated Glu-plasminogen and uPA (0.1 to 1 IU/ml) the degradation of [¹²⁵I]fibrin was 1.5- to 2-fold higher than incubations using nontreated Glu-plasminogen (Fig. 4).

To investigate stachybotrydial-plasminogen interaction, radiolabeled stachybotrydial was produced in a culture of *Stachybotrys* sp. F1839 supplemented with [³H]acetate. The activity of the purified [³H]stachybotrydial in enhancing Glu-plasminogen activation was comparable to the activity of unlabeled stachybotrydial. When [³H]stachybotrydial (120 μ M) was incubated with 3.0 μ M Glu-plasminogen for 20~40 minutes, 1 mole of Glu-plasminogen bound 1.2~1.6 moles of [³H]stachybotrydial (Fig. 5A). Along with the stachybotrydial binding, the rate of tPA-catalyzed activation of the treated Glu-plasminogen increased in a

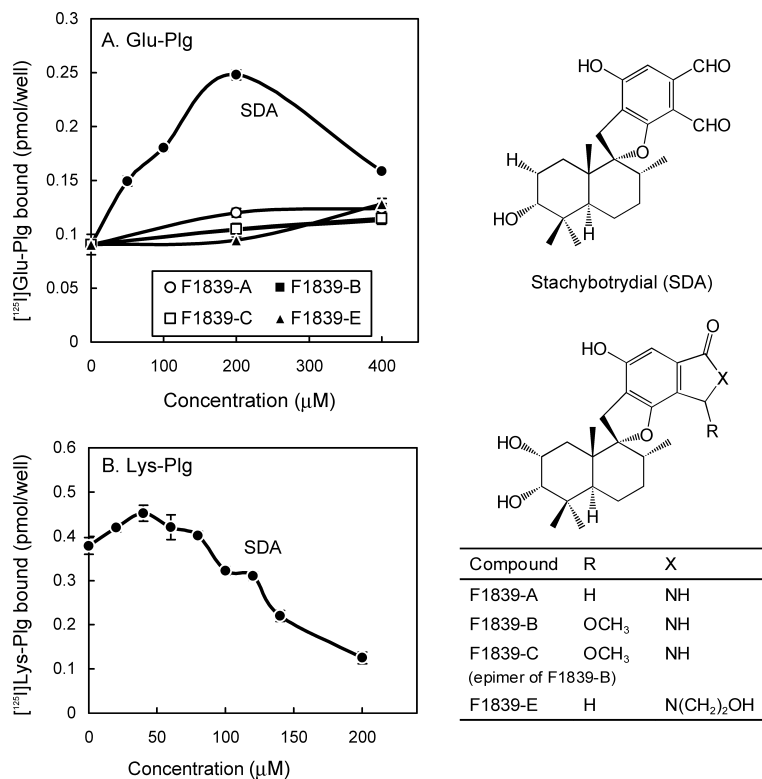


Fig. 1 Effects of stachybotrydial and its analogs on fibrin binding of [¹²⁵I]plasminogen.

The binding of [¹²⁵I]Glu-plasminogen (Glu-Plg) (A) and [¹²⁵I]Lys-plasminogen (Lys-Plg) (B) to fibrin was determined in the presence of the indicated concentrations of the compounds shown in the *right*. The specific binding values, which was calculated by subtracting values obtained in the presence of 20 mM 6-aminohexanoic acid (nonspecific binding) from values obtained in its absence (total binding), are shown. Each value represents the mean ± S.D. from triplicate determinations.

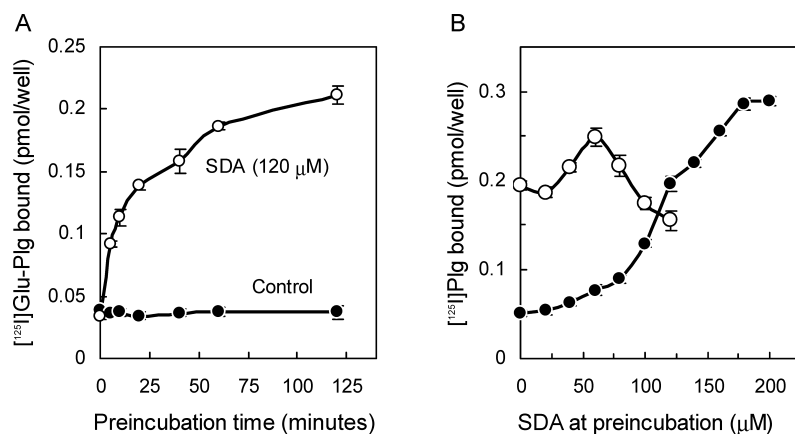


Fig. 2 Effects of stachybotrydial-pretreatment on fibrin binding of [¹²⁵I]plasminogen.

(A) [¹²⁵I]Glu-plasminogen (3.0 μM) was preincubated at 37°C for the indicated times in the absence (●) and presence (○) of 120 μM stachybotrydial (SDA). Subsequently, the mixture was diluted 30-fold with buffer D and assayed for binding of the pretreated [¹²⁵I]Glu-plasminogen to fibrin. (B) [¹²⁵I]Glu-plasminogen (●) (3.0 μM) or [¹²⁵I]Lys-plasminogen (○) (3.0 μM) was preincubated at 37°C for 20 minutes with the indicated concentrations of stachybotrydial, diluted 30-fold with buffer D and assayed for binding of the pretreated [¹²⁵I]-plasminogen to fibrin. The specific binding values are shown. Each value represents the mean ± S.D. from triplicate determinations.

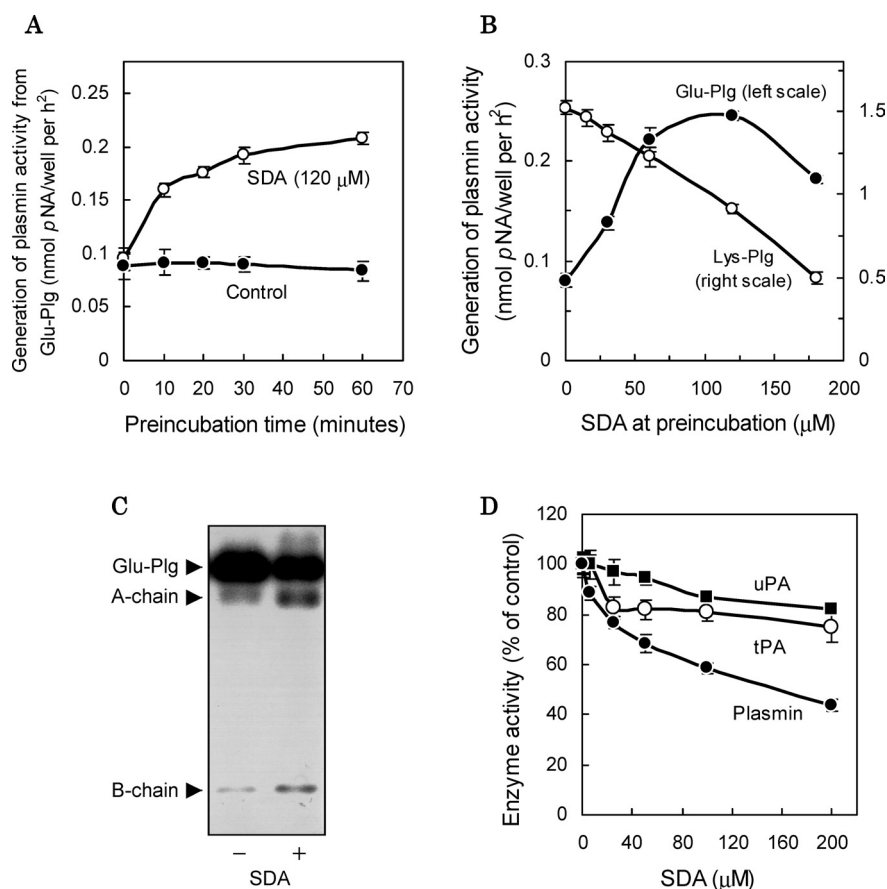


Fig. 3 Effects of stachybotrydial on tPA-catalyzed activation of plasminogen and amidolytic activities of tPA, uPA and plasmin.

(A) Glu-plasminogen ($3.0\ \mu\text{M}$) was preincubated at 37°C for the indicated times in the absence (●) or presence (○) of $120\ \mu\text{M}$ stachybotrydial. Subsequently, the mixture was diluted 30-fold with buffer C and assayed for plasminogen activation in the presence of tPA. (B) Glu-plasminogen (●) ($3.0\ \mu\text{M}$) or Lys-plasminogen (○) ($3.0\ \mu\text{M}$) was preincubated at 37°C for 20 minutes with the indicated concentrations of stachybotrydial, diluted 30-fold with buffer C and assayed for plasminogen activation by tPA. Each value represents the mean \pm S.D. from triplicate determinations. (C) [^{125}I]Glu-plasminogen ($3.0\ \mu\text{M}$) was preincubated at 37°C for 20 minutes in the absence or presence of $120\ \mu\text{M}$ stachybotrydial. Subsequently, the mixture was diluted 30-fold and assayed for tPA-catalyzed conversion of [^{125}I]Glu-plasminogen to [^{125}I]plasmin using SDS-gel electrophoresis. Representative autoradiogram is shown. (D) Amidolytic activities of tPA, uPA and plasmin were determined in the presence of the indicated concentrations of stachybotrydial. Each value represents the mean \pm S.D. from triplicate determinations.

similar pattern (Fig. 5A). These results suggested that a stoichiometric binding of stachybotrydial caused Glu-plasminogen to be more susceptible to activation. Lys-plasminogen also bound [^3H]stachybotrydial with a level comparable to that of Glu-plasminogen (Fig. 5B), while activation of stachybotrydial-bound Lys-plasminogen was not elevated (see Fig. 3B).

Discussion

The present results demonstrate that stachybotrydial enhances fibrin binding and activation of Glu-plasminogen.

Four stachybotrydial analogs, which have in common a *spiro*[4-hydroxy-benzofuran-2(3*H*),1'-6'-hydroxy-2',5',5',8'a-tetramethyl-decahydronaphthalene] moiety as a core structure, are far less potent than stachybotrydial. The difference between stachybotrydial and the inactive compounds is the presence of an aromatic dialdehyde function in stachybotrydial, and it is likely that this structure is essential to the stachybotrydial action. The stachybotrydial effects may be due to an interaction of the agent with Glu-plasminogen from the following observations: (i) the pretreatment of Glu-plasminogen with stachybotrydial results in increases both in fibrin binding and activation of Glu-plasminogen; (ii) stachybotrydial

enhances plasmin conversion of Glu-plasminogen without increasing catalytic activity of tPA or uPA; (iii) stachybotrydial can bind to Glu-plasminogen, and the binding at a molar ratio of 1:1 is sufficient for exerting the effect. A covalent linkage may be involved in the stachybotrydial-plasminogen binding, because acetone

washing does not dissociate [^3H]stachybotrydial from Glu-plasminogen. The aromatic dialdehyde function in the stachybotrydial molecule may play a role in adduct formation with primary amines (*e.g.*, ϵ -amino group of lysine residue) in plasminogen, while the site of modification remains to be determined.

Stachybotrydial also binds to Lys-plasminogen, but stachybotrydial-binding to Lys-plasminogen does not enhance fibrin binding or activation of Lys-plasminogen. The structural difference between Glu-plasminogen and Lys-plasminogen is that Lys-plasminogen lacks the *N*-terminal peptide. The *N*-terminal peptide of Glu-plasminogen interacts with kringle 5 domain in plasminogen and, hence, contributes to maintaining a tight conformation, which is related to a resistance of Glu-plasminogen to activation [14, 15, 38]. Therefore, it seems likely that the stachybotrydial effect is related to a change in the conformational status of Glu-plasminogen. There is a possibility that the binding of stachybotrydial to Glu-plasminogen appears to result in it adopting a conformation similar to Lys-plasminogen. If so, the fibrin binding and activation of stachybotrydial-treated Glu-plasminogen should be comparable to those of Lys-plasminogen. The data in Fig. 2B show that the level of fibrin binding of stachybotrydial-treated Glu-plasminogen is comparable to that of Lys-plasminogen. On the other hand, the activation of stachybotrydial-treated Glu-plasminogen is elevated by 3-fold as compared with nontreated Glu-plasminogen, but the level is 1/6 the level of the activation of Lys-plasminogen (Fig. 3B). These results suggest that, although

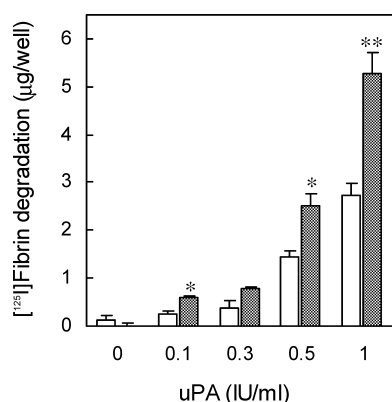


Fig. 4 Fibrinolytic activity of stachybotrydial-treated Glu-plasminogen.

Glu-plasminogen ($3.0\ \mu\text{M}$) was incubated at 37°C for 30 minutes either in the absence (opened bar) or presence (closed bar) of $60\ \mu\text{M}$ stachybotrydial. Subsequently, the treated Glu-plasminogen was diluted 30-fold with buffer C and incubated with [^{125}I]fibrin plate in the presence of the indicated concentrations of uPA to determine fibrin degradation. Each value represents the mean \pm S.D. from triplicate determinations. * and **, $P < 0.05$ and $P < 0.01$ as compared with control (preincubation in the absence of stachybotrydial) by Student's *t*-test.

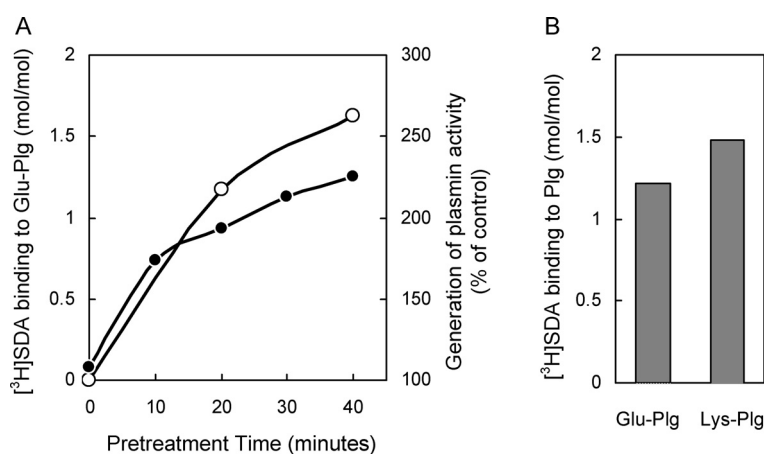


Fig. 5 Binding of [^3H]stachybotrydial to plasminogen.

(A) Glu-plasminogen ($3.0\ \mu\text{M}$) was incubated with either [^3H]stachybotrydial ($120\ \mu\text{M}$) or nonlabeled stachybotrydial ($120\ \mu\text{M}$) at 37°C for the indicated times. Subsequently, the amount of [^3H]stachybotrydial bound was determined in duplicate (\circ). tPA-catalyzed activation of nonlabeled stachybotrydial-treated Glu-plasminogen was determined in triplicate after 30-fold dilution (\bullet). (B) Glu-plasminogen ($3.0\ \mu\text{M}$) or Lys-plasminogen ($3.0\ \mu\text{M}$) was incubated with $120\ \mu\text{M}$ [^3H]stachybotrydial at 37°C for 20 minutes, and [^3H]stachybotrydial binding was determined in duplicate.

stachybotrydial modulates the conformation of Glu-plasminogen, the resulting conformation is somewhat different from that of Lys-plasminogen.

Lysine analogs, such as 6-aminohexanoic acid, bind to the lysine binding sites in Glu-plasminogen and induces a large-scale conformational change in Glu-plasminogen [13, 19, 20]. This is mainly attributed by the disruption of an intramolecular interaction between a lysine residue(s) in the *N*-terminal peptide and the lysine-binding site in kringle 5 domain. Although lysine analogs facilitate the activation of Glu-plasminogen through such a mechanism, these are inhibitory to fibrinolysis because lysine analogs inhibit plasminogen-fibrin binding, which is essential for fibrinolysis. With respect to induction of conformational change in Glu-plasminogen, stachybotrydial and other nonlysine-analogs (staplabin/SMTs and thioplabin) are similar to lysine analogs. In contrast to lysine analogs, stachybotrydial and other nonlysine-analog plasminogen modulators enhance plasminogen-fibrin binding and fibrinolysis. Furthermore, stachybotrydial, which exhibits Glu-plasminogen-specific activity, is distinct from staplabin/SMTs and thioplabin, which enhance fibrin binding and activation of both Glu-plasminogen and Lys-plasminogen [27–29]. Thus, stachybotrydial represent a unique class of nonlysine-analog plasminogen modulator.

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