Effects of penicillins and 6-aminohexanoic acid on the kinetics of human plasmin

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Human plasmin activity is inhibited by various penicillins in a dose-dependent manner. Ampicillin and cloxacillin produce a 50 % inhibition of the globinolytic activity of plasmin at 4.5 and 5.3 mM respectively. A lower inhibitory capacity is displayed by carbenicillin. Assay of plasmin by its amidolytic activity on D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride showed that ampicillin at a concentration producing half-maximal inhibition converted the hyperbolic activity–substrate concentration curve into a sigmoidal curve. A similar conversion occurred in the presence of ampicillin when plasmin was assayed with an alternative chromogenic substrate, L-pyroglutamyl-glycyl-L-arginine *p*-nitroanilide hydrochloride. 6-Aminohexanoic acid at 7.5 μ M abolished the inhibition of plasmin induced by ampicillin. The present observations suggest that ampicillin interacts with plasmin at a regulatory site different from the active site of the enzyme. The effect of 6-aminohexanoic acid indicates that the lysine-binding site may be part of a regulatory site. It is possible that modulation of plasmin activity by ligands plays a role in the control of fibrinolysis.

INTRODUCTION

Plasminogen activators are involved in fibrinolysis and various other biological processes [1–4]. The specific function of plasminogen activator is to convert the inactive zymogen plasminogen into the active proteolytic enzyme, plasmin. It is widely accepted that the control of fibrinolytic activity is exerted at the level of plasminogen activation. Therefore it is not surprising that plasminogen activator activity is under stringent metabolic control and can be modified by a variety of metabolites, inhibitors [5] and hormones [6,7].

We have recently observed that various penicillins inhibit urokinase activity [8] and tissue-type plasminogen activator [9]. Following these studies we noted that the β -lactam compounds ampicillin and cloxacillin can modulate plasmin activity.

The present study shows that several penicillins modify human plasmin activity, suggesting that control of fibrinolysis may also be exerted through regulation of plasmin activity.

MATERIALS AND METHODS

Materials

14 C-labelled globin was prepared by carbamoylation of haemoglobin with $K^{14}CNO$, followed by separation of globin from haem, as previously described [10]. The chromogenic substrates S-2251 (D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride) and S-2444 (L-pyroglutamyl-glycyl-L-arginine *p*-nitroanilide hydrochloride) were from Kabi Diagnostics, Stockholm, Sweden. The plasmin was a commercially available product from Kabi, produced by activation of human Glu-plasminogen in the presence of 6-aminohexanoic acid. Polyacrylamide-gel-electrophoretic analysis showed a single protein of

approx. 80 kDa, corresponding to Lys-plasmin. 6-Aminohexanoic acid was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cloxacillin, ampicillin and carbenicillin were obtained from Teva, Jerusalem, Israel. Ampicillin was diluted in phosphate-buffered saline (120 mM-NaCl/2.7 mM-KCl/10 mM-sodium phosphate buffer, pH 7.4) to a concentration of 200 mg/ml and was used only after storage for 1 month at 4 °C. This storage is known to polymerize the compound [11]. We noted that only stored ampicillin was active as an inhibitor of plasmin.

Methods

Globinolytic assay of plasmin activity. This assay monitors the cleavage of 14 C-labelled globin to acid-soluble products [12]. A portion (0.005 casein unit; [12a]) of plasmin was incubated in 0.4 ml of phosphate-buffered saline with 25 μ l of 14 C-labelled globin (16 mg/ml; 6×10^5 c.p.m./mg) for 30 min at 37 °C. The reaction was terminated by addition of 250 μ l of 50 % (w/w) trichloroacetic acid. After 15 min in an ice/water bath the tubes were centrifuged at 5000 *g* in a refrigerated centrifuge for 15 min. Portions of the supernatant were added to a solution of 40 % (v/v) Lumax (Lumac Systems, Titusville, FL, U.S.A.) in toluene and radioactivity was determined in a liquid-scintillation spectrometer.

Chromogenic assay of plasmin activity. Substrates S-2251 and S-2444 were used to assay the amidolytic activity of plasmin by measurement of the release of *p*-nitroaniline. A portion (0.005 casein unit) of plasmin was incubated in 0.9 ml of phosphate-buffered saline with various substrate concentrations and 0.1 ml of poly(ethylene glycol) (average molecular mass 6000 Da)

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Fig. 1. Effect of penicillins on globinolitic activity of plasmin

Plasmin (0.005 casein unit) was incubated with various concentrations of ampicillin ($\square$), cloxacillin ($\triangle$) and carbenicillin ($\circ$). 100% activity of plasmin was 28 μ g of globin/h. For details see the Materials and methods section. The experiment was repeated three times with essentially similar results, but only one representative experiment is shown.

(5 g/l). The rate of change of absorbance was monitored at 405 nm in a Gilford 250 spectrophotometer. Usually the reaction was monitored for up to 10 min and rates were calculated for the linear portion of activity curves. Minimal change in absorbance over the first minute was not less than 0.025 A unit. Penicillins were added to the buffer at concentrations indicated in the appropriate Figures and legends.

RESULTS

The effect of several penicillins on the globinolitic activity of plasmin is shown in Fig. 1. By using this assay, which monitors the cleavage of ^{14}C -labelled globin to acid-soluble products, 0.005 casein unit of plasmin was found to cleave 28 μ g of globin/h (100%). The presence of various penicillins caused a dose-dependent inhibition of the globinolitic activity of plasmin. The largest inhibition was noted with ampicillin and cloxacillin, which produced half-maximal inhibition at 4.5 ± 0.5 and 5.3 ± 0.7 mM (means $\pm$ S.E.M., $n = 3$) respectively. Carbenicillin had a smaller inhibitory effect, with half-maximal inhibition at 63 mM.

Fig. 2 shows plasmin activity as a function of increasing concentration of the substrate (v versus $[S]$). In these experiments a chromogenic amidolytic assay utilizing the substrate S-2251 was employed to measure initial rates of plasmin activity. In the absence of any modulator and at saturating substrate concentrations, 0.005 casein unit of plasmin cleaved 1.1 nmol of this substrate/min. Addition of ampicillin at a concentration of 4.5 mM caused conversion of the hyperbolic curve into a sigmoidal curve. At a higher concentration of ampicillin (7.5 mM) the sigmoidal curve was further shifted to the right (Fig. 2). When the data from Fig. 2 were converted into a reciprocal plot (Fig. 3), a non-linear curve was obtained in the presence of ampicillin, whereas the control in the absence of the antibiotic produced a straight line, with a calculated apparent K_m value of 0.200 ± 0.007 mM (mean $\pm$ S.E.M., $n = 3$).

Fig. 2. Effect of ampicillin on the amidolytic activity of plasmin with S-2251 as substrate

Various concentrations of the chromogenic substrate S-2251 were incubated with plasmin (0.005 casein unit) in the absence ($\circ$) or in the presence of 4.5 mM- ($\bullet$) and 7.5 mM- ampicillin ($\square$). Cleavage of the substrate was monitored at 405 nm as described in the Materials and methods section. The experiment was repeated three times with essentially similar results, but only one representative experiment is shown.

Fig. 3. Reciprocal plot of the inhibition of plasmin by ampicillin with the chromogenic substrate S-2251

The points were calculated from data presented in Fig. 2. A non-linear curve ($\circ$) was obtained in the presence of 4.5 mM-ampicillin and a straight line ($\bullet$) was obtained in the absence of the antibiotic, with a calculated apparent K_m value of 0.2 mM for the chromogenic substrate.

To exclude the possibility that the sigmoidal curve obtained was due to interference with binding of ampicillin to the lysine-binding site by the lysine residue in the substrate S-2251, an alternative substrate without lysine (namely S-2444) was used. Plots of v versus $[S]$ for plasmin activity with this substrate showed that 6 mM-ampicillin transformed the straight line into a non-linear curve in a reciprocal plot (Fig. 4).

Fig. 4. Reciprocal plot of the inhibition of plasmin by ampicillin with the chromogenic substrate S-2444

Various concentrations of S-2444 were incubated with plasmin (0.005 casein unit) in the absence (●) or in the presence (○) of 6 mM-ampicillin. Cleavage of the substrate was monitored at 405 nm as described in the Materials and methods section. One out of three representative experiments is shown.

Fig. 5. Effect of 6-aminohexanoic acid on the inhibition of plasmin activity by ampicillin

Plasmin (0.005 casein unit), was incubated with 0.2 mM-S-2251 and various concentration of 6-aminohexanoic acid in the absence (○) or in the presence (●) of 4.5 mM-ampicillin.

As assessed by using the chromogenic assay method, the inhibition of plasmin activity exerted by ampicillin was almost abolished in the presence of 6-aminohexanoic acid (Fig. 5). At 7.5 μ M-6-aminohexanoic acid 98–103 % of the plasmin activity was recovered, despite the presence

of 4.5 mM-ampicillin. In the absence of the inhibitor, 6-aminohexanoic acid alone at a concentration of 5 μ M produced a stimulation of plasmin activity of about 6 %.

DISCUSSION

The results of the present study demonstrate an inhibitory effect of ampicillin on plasmin activity. Ampicillin inhibition, demonstrated with two different substrates of plasmin (S-2251 and S-2444), resulted in typical sigmoidal or non-linear reciprocal curves as shown in Figs. 2, 3 and 4. The fact that storage of ampicillin is required for its effect in the present system might be related to polymerization of the compound. The polymerization results in improved binding of ampicillin to proteins [13].

Sigmoidal curves of plasmin activity versus substrate concentration suggest that the effect of the inhibitors may be on a site different from the active site; this concept is supported by the observation that the inhibition exerted by ampicillin is abolished by 6-aminohexanoic acid. 6-Aminohexanoic acid was found to bind specifically and with high affinity to lysine-binding sites [14]; the presence of lysine-binding sites on plasmin and plasminogen that are distant and different from the active site of the enzyme has been documented [15]. Furthermore, conformational changes of plasminogen as a consequence of binding of ligands to lysine-binding sites have been demonstrated [16,17]. The present findings on the ability of 6-aminohexanoic acid to abolish the inhibition by penicillins suggest that the modulators may act through the lysine-binding sites of plasmin. It is noteworthy that 6-aminohexanoic acid alone had no effect on plasmin activity on S-2251. This result supports the contention that the lysine residue of the substrate is not involved in its cleavage by plasmin. A similar conclusion can be drawn from the finding that both a lysine-containing and a non-lysine-containing substrate displayed similar sigmoidal curves.

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