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SIGNIFICANCE OF DIRECT AND INDIRECT KININ FORMATION BY PLASMIN IN HUMAN PLASMA

G. SEIDEL, H.-U. STÜCKER and W. VOGT

Department of Biochemical Pharmacology, Max-Planck-Institut für experimentelle Medizin, Göttingen, Germany

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Abstract—Human plasma was treated with *p*-iodobenzoate to inactivate plasmin inhibitors. Incubation of such plasma with streptokinase generated plasmin activity, detectable by caseinolysis. Simultaneously plasma kinin was liberated. Both lima bean inhibitor which specifically inhibits plasmin, and ε-amino caproic acid which prevents plasmin activation suppressed the formation of kinin, indicating that plasmin was involved in the streptokinase-induced kinin liberation. Plasma heated to 61° at pH 4 still developed caseinolytic activity upon activation with streptokinase, but no kinin formed. Lima bean inhibitor added to non-heated plasma after treatment with *p*-iodobenzoate and streptokinase did not block kinin-forming activity. These findings demonstrate that plasmin formed kinin only indirectly by activating a heat labile plasma kininogenase. This enzyme has properties of kininogenase I, equal to classical plasma kallikrein. At concentrations corresponding to those found in iodobenzoate-treated, streptokinase-activated human plasma, purified human plasmin did not liberate kinin from human kininogen I. Only when added at considerably higher concentrations was a direct kinin formation seen. It is concluded that plasmin acts both as a plasma kallikrein activator and as a kininogenase proper. However, under physiological conditions it probably forms kinin only indirectly.

PLASMA kinins are cleaved from their precursors, kininogens, by specific enzymes like the various kallikreins, or by less specific proteases such as trypsin. Kallikrein(s) of plasma circulate(s) as inactive pre-enzyme(s) which can be activated by various processes, among them activation by proteolytic enzymes which thus induce kinin formation indirectly (for review see Ref. 1).

One of the best known proteolytic enzymes occurring in plasma is plasmin. It has been considered as a kinin-forming enzyme first by Beraldo.[2] Lewis,[3] in studies with a pseudoglobulin fraction of dog plasma, found that plasmin-induced kinin formation followed a typical slow time course, in contrast to the quick action of plasma kallikrein. He concluded that the two enzymes were different kininogenases. In contrast, other authors did not observe kinin formation after incubation of substrate with plasmin.[4–8] The suspicion arose that incubation mixtures in which kinin activity developed were contaminated with kallikrein. The divergent results seemed to be reconciled when it was found that dog pseudoglobulin fractions produced kinins on incubation with plasmin only if plasma prekallikrein capable of undergoing activation was present.[9] This indicated that plasmin acted only indirectly, by activating plasma kallikrein. A kallikrein activator function of plasmin was suggested already by findings of Webster and Pierce,[5] Back *et al.*[10] and Eisen.[11] The indirect kinin formation triggered by plasmin has been confirmed.[8,12]

Later, however, direct kinin formation by plasmin has again been described in incubation mixtures of kininogens and plasmin which could not possibly contain specific pre-kininogenase.[13–15] Thus the reason for the divergent results about kinin formation by plasmin was open to question again. The present experiments were performed in an attempt to find an explanation and to reconcile the various findings. Because of the practical relevance human plasma was used both as substrate and as source of plasmin. In addition some observations were made on the release of kinin from a preparation of human plasma kininogen I by highly purified human plasmin. From the results the possible significance of direct and indirect kinin formation by plasmin, in whole plasma, was assessed.

MATERIALS AND METHODS

Human blood was taken from the antecubital vein through a polyethylene cannula. It was centrifuged in polyethylene tubes after addition of sodium citrate (0·02 ml 20% solution/ml). All manipulations with plasma were carried out in vessels and pipettes made of plastic material.

Preparations

Kininogen I and acid activated plasma ("plasma kallikrein") were prepared according to Vogt and Wawretschek.[16]

Plasma, free of kininogen II ("B-depleted plasma"), was prepared by rotation with glass beads (170 mg/ml; 0·1 mm dia.; 5 min, 37°), decantation and standing for 1 hr at room temperature to allow for decay of liberated kinins.[17,18]

Substances

Purified human plasmin = Forschungs-Plasmin Kabi, 3 u/mg protein, Deutsche Kabi GmbH, München. Streptokinase (Streptase, Behringwerke, Marburg) was kept as a stock solution of 1000 u/ml in 0·9% NaCl at −20°.

Protease inhibitors: ε-aminocaproic acid, lima bean inhibitor (LBI), both from Mann-Research, New York.

p-Iodobenzoic acid (EGA-Chemie, Steinheim, Germany) was purified by repeated crystallisation and sublimation.

Casein, biochemical grade, was purchased from Merck AG, Darmstadt. For further purification a 5% solution in 0·1 N NaOH was precipitated with an equal volume of 10% trichloroacetic acid. The precipitate was redissolved in 0·1 NaOH while standing in a boiling water bath for 15 min. After filtration the solution was dialysed against deionized water. The retentate was lyophilised. The casein preparation was dissolved in 0·1 M phosphate buffer, pH 7·4 to give a 6% solution. The pH was adjusted to 7·4 with 0·1 N NaOH. The final solution was stored at −20°.

Purified hog pancreatic kallikrein was a gift from Dr. D. Auhagen, Farbenfabriken Bayer, Wuppertal-Elberfeld. Bradykinin was a synthetic product kindly supplied by Dr. E. Stürmer, Sandoz AG, Basel.

Estimation of plasmin

The estimation of plasmin was carried out by caseinolysis modified after Sgouris *et al.*[19] To 0·5 ml of test solution (e.g. activated plasma) was added 1·5 ml 0·1 M phos-

phate buffer, pH 7·4 and 4 ml casein solution. After mixing and after 60 min incubation at 37° portions of 2 ml were taken and precipitated with 2 ml 10% trichloroacetic acid. The supernatant was filtered after 20 min, and in 0·2 ml of the filtrate liberated tyrosine was estimated by a modified Folin reaction.[20] Incubations of casein with varying amounts of purified human plasmin were used for calibration. Activities are given as Sgouris units (1 unit = the amount liberating 450 μg acid-soluble tyrosine).

Estimation of kinins

Kinins were estimated by bioassay on the isolated guinea-pig ileum with synthetic bradykinin as standard.

RESULTS

Activation and stabilization of plasmin. Demonstration of kinin formation in plasma

Among other possibilities plasminogen can be activated in human plasma by streptokinase. Once converted to the active state the plasmin is inactivated by binding to inhibitors, at a considerable speed and efficiency. In order to provide optimal conditions for any actions of plasmin in whole plasma methods for preserving its activity were looked for. Von Kaulla[21,22] found that *p*-iodobenzoic acid destroys plasmin inhibitors.

Treatment of plasma with the sodium salt of this acid (PIBA; final concentration 0·074 M) for 30 min at 37° had no adverse effect on subsequent kinin formation induced by glass contact or by trypsin. When plasma pretreated with PIBA was incubated with streptokinase (100 units/ml) both kinin and caseinolytic activities developed (Table 1). No comparable activities appeared when fresh plasma was incubated with streptokinase. On the other hand the treatment with PIBA alone was also not sufficient to generate significant caseinolysis or measurable kinin activity (Table 1). In view of the specificity of streptokinase these results demonstrate that the enzyme activated during the double treatment of plasma and implicated in the formation of kinin was plasmin, and that it could be widely protected from inactivation by PIBA.

Streptokinase was most efficient in activating the kinin-forming process in the range of 100 units/ml plasma. At concentrations 10 times lower or higher the kinin activity induced was definitely lower, in accordance with earlier findings of Eisen.[11] Prolongation of the pre-incubation time with PIBA up to 3 hr or of the incubation with streptokinase increased the kinin production by about 25–30 per cent. Such time consuming incubations thus had no marked effect, but they increased the danger of inadvertently activating other pathways to kinin formation.

As a routine procedure for the activation and stabilization of plasmin in human plasma the following method was therefore adopted: Plasma was mixed with 0·1 ml/ml 1% *o*-phenanthroline–HCl (except in cases where kinin formed should decay), and with 0·1 ml/ml 0·74 M PIBA and incubated for 30 min at 37°. Then 0·1 ml streptokinase solution (1000 units/ml) was added per ml plasma and the mixture was incubated for another 30 min at 37° (or for varying times in kinetic measurements). The values of Table 1 were obtained by this technique.

Plasma which had been activated to liberate kinin by treatment with glass beads ("B-depleted plasma"[17,18]) did also release new kinin when treated with PIBA and streptokinase. In such plasma no kinin formation could be induced by a second glass

TABLE 1. PLASMIN ACTIVITY AND KININ FORMATION IN HUMAN PLASMA PRETREATED WITH *p*-IODOBENZOATE AND ACTIVATED WITH STREPTOKINASE

Experiment no.	Plasmin (u/ml plasma)	Bradykinin equivalents (μg/ml plasma)
1	0·43	1·09
2	1·02	0·93
3	0·79	1·67
4	0·24	1·16
5	0·73	0·65
6	0·46	1·10
7	0·48	1·16
8	0·62	1·56
9	0·70	1·90
10	0·12	0·99
11	0·41	1·27
Mean value ± S.D.	0·55 ± 0·25	1·23 ± 0·36
Plasma without streptokinase activation (n = 11)	0·04 ± 0·04	< 0·11
Plasma without *p*-iodobenzoate pretreatment (n = 8)	0·06 ± 0·02	< 0·09

For details see text.

contact. This means that the glass-treated plasma was free of kininogen II[23] and consequently that the activation of plasmin lead to kinin production from kininogen I.

Effect of inhibitors

ϵ-Aminocaproic acid (ϵ-ACA), an inhibitor of plasmin activation was added to PIBA-treated plasma (final concentration 0·1 M). Then the plasma was incubated with streptokinase and phenanthroline as usual. No measurable kinin activity developed, even after 1 hr incubation (<0·15 μg/ml plasma; n = 2). Subsequent treatment

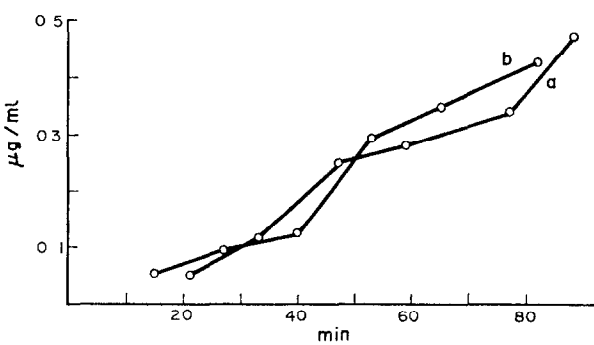

FIG. 1. Formation of kinin during incubation, at 37°, of kininogen I with acid-treated human plasma ("plasma kallikrein") (1 + 1 vol.); (a) in the presence of LBI (1 mg/ml), (b) without LBI. 1 mg/ml phenanthroline present in all incubation mixtures. Ordinate: Bradykinin equivalents in μg per ml added kininogen I preparation. Abscissa: Incubation time in min.

of portions of these samples with glass or with pancreatic kallikrein yielded 0·5 μg/ml and more than 2 μg/ml bradykinin equivalents, respectively, demonstrating that kininogen and even the complete contact system was present.

LBI has been reported by Back and Steger[24] to block plasmin specifically but not plasma kallikrein. It inhibited kinin formation. PIBA-treated plasma was incubated with 0·5 mg/ml ($n = 2$) or 1 mg/ml ($n = 3$) LBI, phenanthroline and streptokinase. No measurable kinin activity developed in any sample ($<0·1$ μg/ml). In control incubations of the same plasma specimen without LBI 0·7–2·4 μg kinin/ml were released.

At the concentrations used LBI did not reduce the kinin forming potency of human plasma kallikrein such as present in acid-treated plasma. One ml plasma kallikrein preparation was treated with 1 mg LBI and was incubated with 1 ml kininogen I solution in the presence of phenanthroline. Controls had no LBI. No difference in kinin formation was apparent ($n = 3$). Figure 1 shows one of these experiments.

The effect of ε-ACA and of LBI again indicates that plasmin is the enzyme activated by the treatment with streptokinase and essential for kinin formation under the experimental conditions chosen.

Demonstration of the indirect action of plasmin

(a) *Selective elimination of (pre-) kallikrein.* In contrast to plasma (pre-) kallikrein plasmin as well as its inactive precursor are fairly heat-stable at low pH.[19] Human plasma, pretreated with PIBA, was adjusted to pH 4 with 0·3 N HCl and then heated to 61° for 1 hr. After cooling it was neutralized with 0·3 N NaOH and incubated with streptokinase. As Fig. 2 demonstrates such plasma exhibited normal plasmin activity

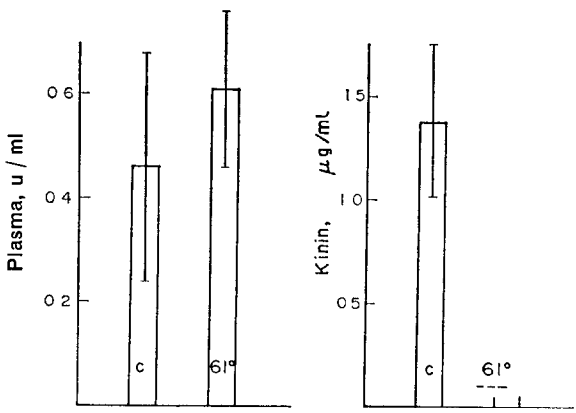

FIG. 2. Comparison between contents of plasmin and kinin in native (C) and heated (61°) plasma (pH 4, 60 min) after incubation with streptokinase (30 min; 37°). Mean values ± S.D.; $n = 5$. Left ordinate: Plasmin in u/ml plasma. Right ordinate: Bradykinin in μg/ml plasma.

as assessed by caseinolysis. Nevertheless the plasma did not liberate kinin. Apparently the plasmin is not sufficient *per se* to release kinin but a heat-labile plasma factor with properties like kallikrein is essential, in addition.

(b) *Selective elimination of plasmin.* Plasma was treated first with PIBA, then for 60–90 min at 37° with streptokinase, in the absence of phenanthroline to allow decay of kinin formed. After 60 min, and if necessary after 90 min again, it was checked whether all biological activity had disappeared. To these samples was then added

TABLE 2. KININ FORMATION (μg/ml ADDED KININOGEN SOLUTION) FROM KININOGEN I INCUBATED WITH HUMAN PLASMA FOR 30 min AT 37°, IN THE PRESENCE OR ABSENCE OF LBI (1 mg/ml)

	Experiment No.		
	1	2	3
With LBI	0·55	0·65	1·54
Without LBI	0·55	0·66	1·54

The plasma had been pretreated with *p*-iodobenzoate, activated by streptokinase and incubated until endogenous kininogen and formed kinin had decayed (90 min, 37°). Phenanthroline was added before the incubation with kininogen I.

LBI (1 mg/ml; saline to the controls) and an equal volume of kininogen I solution. The substrate had also been treated with PIBA and contained LBI in the respective portions. In all of the three experiments performed no difference in kinin formation could be detected, after 60 min incubation (Table 2). In three other experiments of this kind the kinetics of kinin formation were studied. Again no difference between formation in the presence and in the absence of LBI was seen (Fig. 3). Accordingly the plasma samples after their activation contained a kinin forming enzyme which unlike plasmin was not blocked by LBI in the concentrations used and was heat-labile.

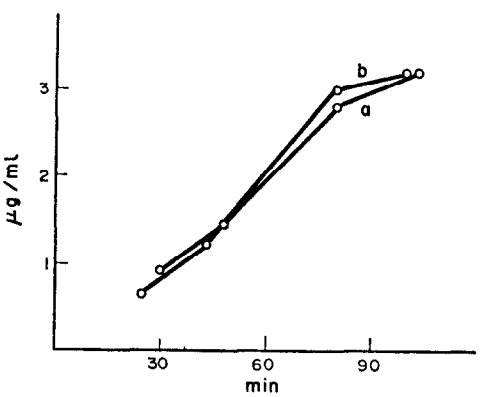

FIG. 3. Formation of kinin during incubation, at 37°, of kinogen I with streptokinase-activated and pre-incubated plasma; (a) in the presence of LBI (0·5 mg/ml), (b) without LBI. Ordinate: Bradykinin in μg/ml added kininogen I preparation. Abscissa: Incubation time in min.

Direct kinin formation by plasmin in purified systems

Kininogen I preparations, some of them pretreated with PIBA, were incubated with purified human plasmin, in the presence of phenanthroline, at 37° for 60 min. No kinin formation was detectable when 1 unit plasmin/ml or less were used. Two to three units/ml produced a slight release only. In order to obtain as much kinin as could be developed with hog pancreatic kallikrein 7 units/ml were necessary. LBI blocked this release (Fig. 4).

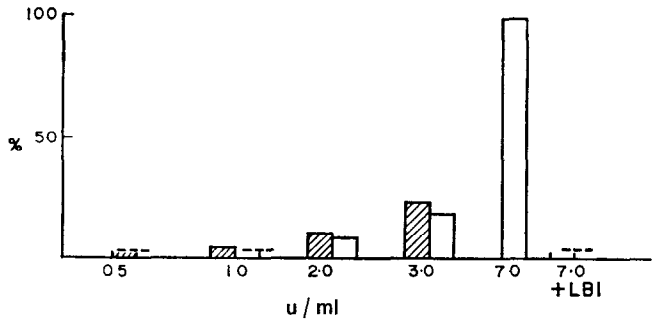

FIG. 4. Formation of kinin from kininogen I by incubation with plasmin (60 min, 37°). Values are given in per cent of release by hog pancreatic kallikrein. Mean of two experiments. Hatched columns: Experiments with *p*-iodobenzoate-pre-incubated kininogen. Open columns: No pretreatment with *p*-iodobenzoate. Last column: Incubation in the presence of 0·64 mg/ml LBI. Plasmin added at concentrations as indicated below columns (u/ml).

DISCUSSION

Treatment of human plasma with streptokinase induced kinin formation provided plasmin inhibitors had been destroyed by PIBA. The implication of plasmin in this process is evident from the inhibition by ε-ACA, by LBI and from the well-known specific activity of streptokinase. On the other hand the activated plasmin is not the kininogenase proper: when the plasma is heated at pH 4°–61° before activation caseinolytic and kinin forming potency dissociate. The latter is abolished whereas the former is unaltered. Conversely, the plasmin activity can be specifically blocked by LBI without impairing kinin formation, once time has been allowed for the plasmin to act on components of its environment—plasma. This means that in these conditions plasmin only activates a plasma kininogenase which in turn releases kinin.

The activated kininogenase is sensitive to moderate heat and acid, and it is not inhibited by LBI, all in contrast to plasmin. It apparently utilizes kininogen I. This follows from the finding that kinin formation by plasmin was also seen in plasma the kininogen II of which had been exhausted by previous contact activation with glass. Thus the activated enzyme has several properties of kininogenase I which is identical with classical serum kallikrein.

The finding that plasmin activates kininogenase I in plasma pretreated with glass requires a revision of the scheme of events happening in human plasma during contact activation, proposed earlier.[23] In order to account for the fact that hardly any kininogenase I activity could be generated by acid, acetone or contact in glass-treated plasma it was assumed that this enzyme had been activated and subsequently inactivated in the course of the glass treatment. It is now evident that pre-kininogenase I is still present but cannot be activated by the processes mentioned probably because of lack of some intermediate link in the activation chain. This problem is now under investigation.

In contrast to the experiments with human plasma in which no indication for a direct kinin releasing effect of endogenous plasmin was detected, purified human plasmin was able to directly cleave kininogen I contained in an enzyme-free plasma preparation. However, rather high concentrations were necessary for the direct kinin formation—7 units/ml. Such concentrations were never found in plasma. It follows that endogenous plasmin will cause kinin release only indirectly, not by a direct

kininogenolytic effect, in accordance with an earlier suggestion.[9] Apparently much lower concentrations of plasmin (0·5–1 unit/ml) are sufficient to activate plasma kallikrein than are necessary for a significant cleavage of kininogen. This agrees with findings of Henriques *et al.*[14] Back and Steger[13] used 20–100 RPMI units plasmin/ml to release kinin directly. This is again much more than was found by these authors in plasma to occur (3–15 RPMI units/ml; personal communication by Dr. N. Back).

Even the indirect kinin formation induced by endogenous plasmin was observed only under optimal, artificial conditions, namely, when plasmin inhibitors had been reduced by PIBA. Under more natural conditions it would seem unlikely that comparable plasmin activities could be attained in the circulating plasma, because of the rapid inactivation. This makes it rather doubtful whether plasmin can cause significant kinin formation in the blood stream, if only indirectly. Only under pathological conditions, in hyperplasminaemia or lack of inhibitors could such a process appear to be possible. Another possibility might be a local kinin formation by action of plasmin activated and incorporated in a clot, where it would be protected from inactivators.[25]

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