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ACTIVATION OF DOG PLASMA KININOGENASE WITH GLASS

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Abstract—The activity of glass-activated kallikreins in dog plasma was determined by measuring both kininogenase and *p*-toluensulphonyl-L-arginine methyl ester (TAME) esterase activities. Non-contact plasma, after being shaken with glass beads, was centrifuged immediately at 4° to sediment the glass beads and the supernatant was used for the experiments. Glass activation of dog plasma was more effective on TAME esterase activity. When the supernatant alone was preincubated at 37°, its kininogenase activity was suppressed about 50 per cent, while TAME esterase activity was unchanged. High kininogenase activity in the supernatant could be induced by treatment with 50% ammonium sulphate, suggesting the presence of peculiar inhibitors for kininogenase activity in glass-activated dog plasma. Kininogenase inhibitors in plasma were separated by gel filtration on Sephadex G-200; there were two inhibitors which were able to suppress kininogenase activity in glass-activated dog plasma. When dog plasma was preincubated with lima bean trypsin inhibitor, generation of both kininogenase and TAME esterase was significantly suppressed during contact with glass beads. Acetone-activated dog plasma could be reactivated by glass contact, although the level of kininogenase activity was lower than that by glass activation alone. The non-absorbed supernatant of glass-activated dog plasma formed kinins from rat kininogen. The present results suggest that the kinin-forming system by glass activation in dog plasma is not qualitatively different from that in human plasma with the exception of the presence of potent kininogenase inhibitors in the plasma.

IT IS KNOWN that dog plasma does not form kinin during contact with glass.[1,2] Vogt[3] suggests the existence of two kinin-forming systems: kininogenase I and II. Vogt also considers that kininogenase II is absent in dog plasma, which contains only the complete system I, since dog plasma forms kinin when incubated with pancreas kallikrein and does not form kinin when put in contact with glass. Hageman factor (HF), which is activated by glass surfaces, initiates plasma kinin formation by activating the precursor of kininogenase.[4] Glass activation of dog plasma is ineffective even if glass beads coated with HF from human plasma are used.[5]

On the other hand, Didisheim *et al.*[6] found HF in dog plasma. Temme *et al.*[7] described a pre-kininogenase which is activated by highly purified bovine HF. Recently Collins *et al.*[8] induced kinin formation by glass-absorbed kininogenases of dog plasma.

The present experiments were performed in an attempt to find a reason for the divergent results on kinin formation in dog plasma, and to investigate activation of dog plasma kininogenase with glass by measuring both kinin forming and esterase activities.

MATERIALS AND METHODS

Blood. Mongrel dogs weighing 15–18 kg were used for the experiments. Blood samples were collected into siliconized syringes (one part of 3·1% sodium citrate dihyd-

rate to nine parts of blood) by vein puncture and centrifuged in polyethylene tubes. The separated plasmas were stored in polyethylene tubes at 4°. Human and rat plasma was obtained also using vein puncture and heart puncture, respectively.

Plasma kallikreins. Fresh plasma was shaken with 0·1 mm diameter glass beads at room temperature for 10 min and then centrifuged at 4°. The amount of glass beads was varied per ml of plasma. The activities of kininogenase and *p*-toluenesulfonyl-L-arginine methyl ester (TAME) esterase were measured in the supernatant.

To eliminate kallikrein inhibitors, the supernatant obtained after glass activation was treated by adding an equal volume of a saturated solution of ammonium sulphate.[9] After centrifugation the precipitate was reconstituted to the original volume with 0·9% saline solution.

Acetone activation of fresh plasma was carried out by adding acetone to plasma (20% v/v of acetone),[10] and the mixture was allowed to stand in polyethylene tubes at room temperature for 17 hr. The acetone was then evaporated in a rotary evaporator.

Kininogen. Kininogen substrate was prepared by the method of Eisen;[11] dog plasma was heated at 60° for 60 min. The precipitate was eliminated by centrifugation.

Gel filtration on Sephadex G-200. Forty ml of supernatant from 20 ml of dog plasma brought to 50 per cent saturation with ammonium sulphate were concentrated about three-fold using an Amicon Ultrafiltration Chamber with a UM-10 membrane. The supernatant was subjected to gel filtration on a 3·5 × 41 cm column of Sephadex G-200. The column had been previously equilibrated with 0·05 M phosphate buffer, pH 7·6, containing 0·15 M NaCl. Elution was performed using the buffer solution above. The flow rate was 15 ml/hr, and 5 ml fractions were collected. The adsorption at 280 nm and the activities kininogenase and TAME esterase in the effluent were measured.

Assay of kinins.[12] Kinins released by the kininogenases were assayed on isolated guinea-pig ileum suspended in a muscle bath containing 10 ml of oxygenated Tyrode's solution using synthetic bradykinin as a standard. The ileum was treated with α-chymotrypsin as described by Edery[13] prior to bioassay.

Estimation of kininogenase activity. To 0·2 ml of heat-treated plasma substrate in polyethylene tubes were added 0·1 or 0·2 ml of activated plasma, 0·1 ml of 1,10-phenanthroline (final concn 10^{-5} M) and 0·5 or 0·6 ml of 0·1 M Tris buffer, pH 7·8. After incubation at 37° for 10 min the reaction was stopped by heating for 5 min in a boiling water bath.[10] The kinin values were expressed as ng bradykinin/ml plasma. Liberated kinins were found to be inactivated by incubation with α-chymotrypsin and resistant to trypsin.

Estimation of esterase activity.[14,15] Esterase activity was measured by determining the methanol released from TAME (final concn 0·015 M) using 0·1 or 0·2 ml plasma samples.

Chemicals. TAME was obtained from the Protein Research Foundation, Osaka, Japan and lima bean trypsin inhibitor (LBTI) from Sigma Chemical Co., U.S.A. (1 mg inhibits about 4 mg trypsin). Bradykinin was kindly supplied by Sandoz, A. G., Basel, Switzerland.

RESULTS

Glass activation of plasma. (1) Amounts of glass beads. Non-contact plasma was shaken with varied amounts of glass beads for 10 min at room temperature and

separated into supernatant and glass beads by centrifugation. Collins *et al.*[8] reported that adsorption onto activating surfaces may change the properties of plasma kininogenases. To circumvent this problem occurring during glass contact, the supernatant was used in the experiments.

TABLE 1. EFFECT OF THE AMOUNT OF GLASS BEADS ON KININOGENASE AND ESTERASE ACTIVITIES IN PLASMA

	Glass beads (mg/ml)				
	0	50	100	200	400
Kininogenase activity (ng bradykinin/ml plasma)	—*	65	70	73	49
TAME esterase activity (μmoles hydrolysed/ml plasma/hr)	<1·2	6·1	6·8	6·9	6·8

* Unmeasurable.

Kininogenase activity developed in a series of the experiments and reached a plateau at 100 mg glass beads per ml plasma. The low effectiveness of glass activation on plasma is considered to be due partly to glass-activated kininogenases being adsorbed on the glass surfaces.[16] TAME esterase activity followed a similar pattern, but the activity was significantly enhanced compared with kininogenase activity (Table 1). From these findings 100 mg of glass beads was used for glass activation.

(2) Treatment with ammonium sulphate. Control samples were always incubated with saline instead of kininogen substrate. Free kinins in 0·2 ml of non-contact plasma were not detected. Free kinins in the supernatant obtained after glass contact were also unmeasurable in the absence of 1,10-phenanthroline, indicating that plasma kininase inactivates endogenous kinins liberated.

TABLE 2. EFFECT OF TREATMENT WITH AMMONIUM SULPHATE ON GLASS-ACTIVATED PLASMA

	Control (non-contact plasma)	Glass-activated plasma	Glass-activated plasma treated with ammonium sulphate
Kininogenase activity (ng bradykinin /ml plasma)	—*	71 ± 21†	149 ± 35‡
TAME esterase activity (μmoles hydrolysed/ml plasma/hr)	<1·2	7·2 ± 0·4§	8·4 ± 0·3

* Unmeasurable.

† Statistical significance of difference between glass-activated plasma and controls: $P < 0.01$.

‡ Statistical significance of difference between glass-activated plasma treated with ammonium sulphate and glass-activated plasma: $P < 0.05$.

§ Statistical significance of difference between glass-activated plasma and controls: $P < 0.001$.

When the supernatants were treated with 50% ammonium sulphate to eliminate kallikrein inhibitors, kininogenase activity significantly increased while TAME esterase activity was increased to a lesser degree (Table 2).

On the other hand, more kinins were liberated in the assay of kininogenase activity in the supernatant using rat kininogen substrate prepared by the same procedure as dog kininogen. Furthermore considerable amounts of kinins were formed in human

plasma by glass treatment alone in comparison with dog plasma as reported also by Rocha e Silva *et al.*[17]

(3) Effect of preincubation. The supernatants were preincubated for 10 and 30 min at 37° and then treated with ammonium sulphate. Controls were preincubated after previous treatment with ammonium sulphate. Kininogenase activity was significantly inhibited, 52 per cent by 10 min preincubation and 54 per cent by 30 min preincubation, while TAME esterase activity was unchanged.

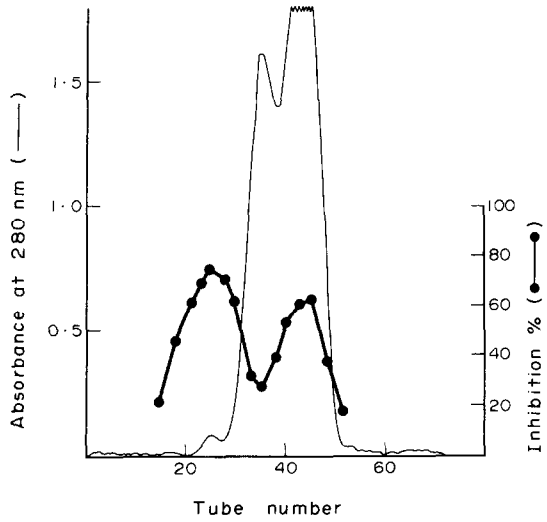

FIG. 1. Gel filtration of plasma kallikrein inhibitor on Sephadex G-200. Plasma treated with ammonium sulphate after glass contact was used as plasma kallikreins.

The results of (2) and (3) suggest that plasma may contain kallikrein inhibitors, which inhibit kininogenase activity of kallikreins more effectively than esterase activity in glass-activated dog plasma.

Gelfiltration of supernatant on Sephadex G-200. Supernatant was dialysed overnight against 0·05 M phosphate buffer, pH 7·8, containing 0·15 M NaCl and concentrated three-fold. About 13 ml of the concentrated supernatant were applied to a Sephadex G-200 column. A typical elution pattern is shown in Fig. 1. For measurement of the inhibitory activity of plasma kininogenase, 0·2 ml of plasma treated with 50% ammonium sulphate followed by glass contact was incubated with 0·4 ml of eluate for 10 min at 37° and kininogenase and esterase activities were estimated.

The kininogenase inhibitory activity was found in two areas; the first was found in fractions 15–35, and the second, in fractions 36–55. No measurable inhibition of TAME esterase activity was observed in each 0·4 ml of eluate.

Inhibition by plasma inhibitors. The eluates in fractions 20–30 and 40–50 were pooled and concentrated by ultrafiltration. The protein content of the solutions was determined by applying the formula of Kalkar.[18] For measurement of inhibitory activity, each fraction was preincubated with 0·2 ml of glass-activated plasma as described above. The final concentration of the inhibitory fractions for kininogenase and esterase assays was 0·05 and 0·5 mg/ml, respectively.

Kininogenase activity was inhibited more than 50% by both inhibitory fractions, while esterase activity was hardly inhibited by a final concentration of 0·5 mg/ml (Table 3). These results support the assumption described in the results of Table 2 that potent inhibitors for glass-activated kininogenase may be present in plasma.

TABLE 3. INHIBITORY ACTIVITY OF KALLIKREIN INHIBITORS SEPARATED FROM PLASMA

	Inhibitory activity (%) of fraction	
	1st	2nd
Kininogenase activity	52 ± 6*	54 ± 4*
TAME esterase activity	0	7 ± 2

Plasma treated with ammonium sulphate after glass contact was used as plasma kallikreins.

* Statistical significance of difference from controls: $P < 0.01$.

Effect of LBTI. Glass beads initiate plasma kininogenase activity by activating HF which in turn activates the precursor of kininogen II.[4,19] LBTI has been reported to inhibit HF strongly but not to inhibit plasma kallikreins.[20,21] LBTI might suppress the activation of the precursor of kininogenase II by inhibiting HF activated during shaking with glass beads. Therefore, non-contact fresh plasmas were preincubated with LBTI over a wide range of concentrations in polyethylene tubes before glass activation, confirming that LBTI up to 500 μg did not inhibit dog plasma kallikreins activated by glass contact. The same amount of LBTI was added to controls after glass activation.

TABLE 4. EFFECT OF LBTI ON GLASS ACTIVATION OF PLASMA

	Inhibition (%) LBTI (final concn μg/ml)			
	0	20	100	500
Kininogenase activity	0	42 ± 4	63 ± 5	72 ± 9*
TAME esterase activity	0	20 ± 2	45 ± 2	62 ± 3*

Varied amounts of LBTI were incubated with non-contact plasma for 10 min at 37°. Immediately after glass activation, plasma was treated with 50% ammonium sulphate.

* Statistical significance of difference from controls: $P < 0.01$.

Kininogenase and esterase activities in glass-activated plasma were suppressed by increasing concentrations of LBTI up to 500 μg/ml, suggesting that LBTI suppresses a pathway related to the activation of kininogenase II by glass beads (Table 4). Similar inhibitory effects were described in the study using highly purified bovine HF for *N*-benzoyl-L-arginine ethyl ester esterase activity.

Effect of acetone. Treatment of plasma with acetone mainly activates the precursor of kininogenase I. Kininogenase and esterase activities were generated by acetone activation, indicating less effectiveness on kininogenase activity and more effectiveness on esterase activity compared with glass activation. This predominant activation of esterase may be caused by activation of not only plasma kallikreins but also other proteolytic enzymes which attack arginine esters.[22]

After acetone activation, plasma was shaken with glass beads as usual. Plasma allowed to stand in polyethylene tubes at room temperature without adding acetone was used as a control. Treatment with ammonium sulphate of acetone and glass-activated plasma was omitted, because acetone can destroy kallikrein inhibitors.[23] Kininogenase and esterase activities in acetone-treated plasma were enhanced further by glass contact, showing additional generation of kallikreins by glass activation (Table 5).

TABLE 5. EFFECT OF GLASS CONTACT ON ACETONE-TREATED PLASMA

	Control (non-contact plasma)	Plasma treated with acetone	Glass-activated plasma after acetone treatment
Kininogenase activity (ng bradykinin /ml plasma)	—*	35 ± 8†	46 ± 10‡
TAME esterase activity (μmoles hydrolysed /ml plasma/hr)	<1·2	17·2 ± 0·6§	21·1 ± 0·7

* Unmeasurable.
† Statistical significance of difference between plasma treated with acetone and controls: $P < 0{\cdot}001$.
‡ Statistical significance of difference between glass-activated plasma after acetone treatment and plasma treated with acetone: $P < 0{\cdot}1$.
§ Statistical significance of difference between plasma treated with acetone and controls: $P < 0{\cdot}001$.

DISCUSSION

To survey, in dogs, the kininogenase system activated by glass contact, the following three factors must be considered in plasma: (1) presence of HF; (2) plasma inhibitors towards glass-activated kininogenase; and (3) presence of kininogenase II defined by Vogt.

It has been reported that the kininogenase system in dog plasma is not activated by glass beads[1,2] although microcrystalline mono-sodium urate activated HF in the synovial fluid in dogs.[24] Enhancement of kininogenase and TAME esterase activities by contact with glass beads (Tables 1 and 2) and suppression of their activities by preincubation with LBTI (Table 4) strongly suggest the presence of HF in plasma. The preincubation of plasma with LBTI might be expected to inhibit the activation by glass of inactive HF to active HF, or the conversion of prokininogenase to kininogenase by active HF, or both. The present findings are in good agreement with the result that HF is found in dog plasma.

Glass activation was found to be more effective in human plasma than in dog plasma in agreement with previous work.[8,17] This species difference may be attributed to the amounts of precursors of kallikreins including intermediate substances and their inhibitors. This may explain why plasma kallikrein inhibitors are less effective on kininogenase activity in human plasma than in dog plasma. The findings that glass-activated kininogenases were significantly inhibited by addition of two kallikrein inhibitors (Table 3) and by preincubation only indicate that glass-activated kininogenases are always influenced by their inhibitors in plasma during or after contact with glass. Inability or less ability of dog plasma to produce kinins when glass-activated plasma was used as an enzyme source may be mainly due to the presence of two potent kininogenase inhibitors in plasma.

It is likely that dog plasma contains kininogenase II since glass contact induced kininogenase activity in dog plasma, in spite of the findings[3] that dog plasma contains only the complete kininogenase system I. From the qualitative aspect, it is meaningful that acetone-activated plasma can be additionally activated by glass contact, although the level of kininogenase activity in the experiments is lower than that of glass activation in Table 2. This finding also indicates the presence of kininogenase II in dog plasma, suggesting that some intermediate substances important for glass activation would still remain in the course of acetone treatment.

There is no kininogen I in rat plasma according to Vogt.[3] Therefore, glass-activated dog plasma should not act on rat kininogen. But the fact that glass-activated dog plasma can form kinins from rat kininogen leads to the conclusion that glass-activated dog plasma contains kininogenase II. Collins *et al.*[8] reported that glass-adsorbed dog kininogenases were effective on rat kininogen and they suggested that adsorption may change the enzyme characteristics of plasma kallikreins. If the presence of kininogenae II in dog plasma is not accepted, it will be difficult to explain why the supernatant, non-adsorbed, glass-activated dog plasma, acted on rat kininogen in the present experiments. The possibility that these systems in plasma are only simulated due to interactions between inhibitors and enzymes was recently described by Vogt's colleagues.[2,5]

In conclusion, the present results suggest that glass activation of the kinin forming system in dog plasma is not qualitatively different from human plasma with the exception of the presence of two potent kininogenase inhibitors.

Whether the two kinin forming systems in dog plasma are both present or not is currently under investigation.

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