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SYNTHESIS OF PREKALLIKREIN AND METABOLISM OF PLASMA KALLIKREIN BY PERFUSED RAT LIVER*

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Abstract—When livers from normal rats were perfused *in situ* with oxygenated Tyrode's solution containing bovine serum albumin, both active kallikrein and prekallikrein could be found in the perfusates. Based on five liver perfusates the average rate of synthesis was $6.6 \pm 2.0\,mU \cdot hr^{-1} \cdot (g\ liver)^{-1}$ when *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide was used as substrate, and it was calculated that within 30 hr each liver could synthesize 50 per cent of the prekallikrein content of rat plasma. The liver was also capable of rapidly inactivating rat and human plasma kallikreins and plasmin but not rat urinary kallikrein. The rates of clearance from perfusate were biphasic, giving an initial rapid clearance (2 min) succeeded by a slower phase that followed an exponential curve. These data clearly show that the exsanguinated and perfused rat liver is capable of synthesizing plasma prekallikrein and of metabolizing plasma kallikrein and plasmin.

Plasma kallikrein has been shown to exist in human plasma as an inactive enzyme precursor called prekallikrein. The enzyme catalyzes the production of the potent vasodilator polypeptide, bradykinin, from its preferred substrate HMW kininogen.‡ There is also *in vitro* evidence that it is required for a normal rate of activation of the intrinsic coagulation system [1] and that it is capable of directly converting plasminogen to plasmin [2] and pro-renin to renin [3–5]. It circulates in normal human plasma bound to HMW kininogen [6].

Little direct evidence is available concerning the site of synthesis of this plasma protein. There is abundant evidence that plasma prekallikrein is diminished in patients with cirrhosis of the liver [7] and that this decrease is most likely caused by a decrease in its synthesis. However, direct evidence that prekallikrein is synthesized by the liver has not yet been demonstrated. While studying kininogen synthesis by livers of normal and injured rats, we observed in two experiments that perfused rat liver produced a small amount of an enzyme that could form kinin when added to heat-denatured rat plasma [8]. In the present report, these preliminary experiments have been extended. Efforts were made to characterize the kinin-releasing activity as well as to study some aspects of the participation of the liver in the synthesis of plasma prekallikrein and the metabolism of active kallikrein.

MATERIALS AND METHODS

Bovine serum albumin (fraction V, Lot No. A-4503), *p*-tosyl-L-arginine methylester (TAME), dextran sulfate, sodium salt *ca.* average mol. wt 500,000 and cycloheximide were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), [^{3}H]TAME (210 Ci/mole), from the Biochemical & Nuclear Corp. (Burbank, CA, U.S.A), Pro-Phe-ArgpNA, *N*-benzoyl-Pro-Phe-ArgpNA, Val-Leu-LyspNA, Val-Leu-ArgpNA and streptokinase from Kabi Diagnostica (Stockholm, Sweden), SBTI from Worthington Biochemical Corp. (Freehold, NJ. U.S.A.), and plasmin from the American National Red Cross (20 casein units/mg). Pure bradykinin was synthesized and purified by Professor A. C. M. Paiva, Department of Biophysics, Escola Paulista de Medicina, São Paulo, Brasil. Antibodies to rat plasma albumin were a gift from Dr. A. H. Gordon, National Institute for Medical Research, Mill Hill, London, England.

Human HMW kininogen was prepared as described by Pierce and Guimarães [9]. The B-4-gamma preparation used in these studies contained 10.9 μg bradykinin/mg. Rat plasma kallikrein was prepared from rat plasma which had been adsorbed onto DEAE–cellulose, as described previously for kininogen [9]. The filtrate from this batch adsorption was purified further by ammonium sulfate precipitation between 33 and 46% saturation and by affinity chromatography on benzamidine–Sepharose [10]. The final product contained 600 $mU/A_{280}nm$ when assayed with Pro-Phe-ArgpNA (0.2 mM) as substrate. Rat plasma active Hageman factor (HFa) was prepared by adsorption of rat plasma to supercel as described by Webster *et al.* [11].

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‡ Abbreviations: Pro-Phe-ArgpNA, H-D-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide; *N*-benzoyl-Pro-Phe-ArgpNA, *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginylp-nitronilide; Val-Leu-LyspNA, H-D-valyl-L-L-lysyl-*p*-nitroanilide; Val-Leu-ArgpNA, H-D-valyl-L-leucyl-L-arginyl-*p*-nitroanilide; SBTI, soybean trypsin inhibitor; [^{3}H]TAME, *p*-tosyl-L-arginine [^{3}H] methylester; and HMW kininogen, high molecular weight kininogen.

Rat urinary kallikrein was prepared from rat urine by adsorption on DEAE–Sephadex A-50 as described previously [12]. The final product contained 8 U/mg protein when assayed with Val-Leu-ArgpNA.

Liver perfusion in situ. Following exsanguination *in situ* by perfusion with 80 ml of Tyrode's solution (gassed with 5% CO_2:95% O_2), the livers of 280–320 g Wistar albino rats were perfused at 37° through the portal vein with 35 ml of recirculating Tyrode's solution that was gassed and contained bovine serum albumin (40 mg/ml). The flow rate was 12–16 ml/min and the perfusion pressure was 10–16 cm of H_2O. Less than 0.1% blood was found in the perfusates, as determined by red blood cell counts. Details of this preparation have been described [13].

Cycloheximide treatment. To inhibit protein synthesis, the rats were injected i.p. with a saline solution (2.5 mg/ml) of cycloheximide (2 mg/kg) 17 h before perfusion and with a second cycloheximide injection (3 mg/kg) 2 h before perfusion.

Esterolytic activity. The abilities of the perfusates to digest arginine esterase were determined by a radiochemical method employing [^{3}H]TAME [14]. In this procedure, 20 μl of the perfusate (concentrated 10-fold by pressure dialysis against 0.1 M sodium phosphate buffer, pH 7.65, containing 0.15 M NaCl), was added to a mixture of 10 μl of 0.5 M Tris, pH 8.0, and either 20 μl of H_2O or 20 μl of SBTI (50 μg/ml) and incubated for 10 min at room temperature. [^{3}H]TAME (10 μl, 0.047 μCi) was added and the incubation was continued for an additional 30 min at room temperature. The [^{3}H]methanol formed was counted in an LS-100 Beckman scintillation counter.

Amidolytic activity. Four chromogenic substrates were employed in these determinations: Val-Leu-LyspNA, a plasmin substrate; Pro-Phe-ArgpNA and *N*-benzoyl-Pro-Phe-ArgpNA, substrates for plasma kallikrein; and Val-Leu-ArgpNA, a substrate for urinary kallikrein [15]. The conditions employed were similar to those described by Ammundsen and Svendsen [16]. For determining the activity of the perfusates, 0.3 ml of the non-concentrated perfusate was incubated for 0, 15, 30 and 60 min at 37° with a 0.2 mM solution of the amide in 0.1 M Tris buffer, pH 8.0, in a final volume of 1.0 ml. In the experiments designed to test the possible presence of prekallikrein in the perfusates, three different methods of activation were employed: (1) 0.1 ml perfusate plus 0.1 ml of 0.04 M Tris–HCl buffer, pH 8.0, plus 30 μl HFa in a final volume of 0.3 ml were incubated for 2 min at room temperature (2) 25 μl of dextran sulfate (50 μg/ml) was substituted for the HFa; (3) 2.0 ml aliquots of the perfusates were acidified to pH 2.0 by the addition of 3 M HCl, kept for 10 min at room temperature, and readjusted to pH 8.0 with 3.5 M NaOH. Following activation, 0.2 ml aliquots were used for the amidolytic assays. For detection of plasminogen, 0.3 ml of perfusate was incubated in the presence or absence of 10 μl of streptokinase solution (5000 E units/ml) for 5 min at 37° prior to the amidolytic assay. One mU corresponds to the release of 1 nmole of *p*-nitroaniline/min at 37° and pH 8.0, measured at 405 nm.

Kinin-releasing activity. Aliquots (2.0 ml) of the unconcentrated perfusates were acidified to pH 2.0 by the addition of 3 M HCl, kept for 10 min at room temperature, and readjusted to pH 8.0 with 3.5 M NaOH. To 0.9 ml of this solution were added 10 μl of 0.1 M Tris, pH 8.0, and 100 μl of pure human HMW kininogen (0.93 mg/ml water), and the solution was incubated at 37°. Samples were removed after 0, 15 and 30 min of incubation and kept at 4° until assayed on the rat uterus against a standard bradykinin solution. One mU is equivalent to the liberation of 1 ng bradykinin/min.

Rat plasma albumin. This protein was determined by immunoassay as described previously [17], employing specific rat anti-albumin. There was no cross reactivity between this antibody and bovine serum albumin.

RESULTS

Amidolytic and kinin-releasing activity of rat liver perfusates. In ten preliminary experiments, perfusates of normal rat liver contained arginine esterases capable of cleaving TAME. SBTI only partially blocked this esterolytic activity, and an increase in this SBTI-resistant esterolytic activity occurred with

Table 1. Synthesis of amidolytic and kinin-releasing enzymes by perfused rat liver

Expt.	Perfusion time (min)	Val-Leu-LyspNA* (mU/g liver) (N = 2)	Pro-Phe-ArgpNA† (mU/g liver) (N = 2)	Kinin-releasing‡ (U/g liver) (N = 2)
A	2	0.2 (0.2; 0.3)	0.4 (0.2; 0.6)	
	30	0.4 (0.1; 0.8)	1.0 (0.5; 1.6)	
	60	0.3 (0.1; 0.5)	3.5 (1.6; 5.4)	
B			(N = 5)	
	2		2.1 ± 1.6	2.2 (1.2; 3.1)
	30		3.7 ± 0.2	ND§
	60		5.9 ± 2.1	3.3 (single determination)
	120		10.0 ± 4.0	10.6 (8.7; 12.5)

* Synthetic plasmin substrate; individual values are in parentheses.

† Synthetic plasma kallikrein substrate; in Experiment A individual values are in parentheses; in Experiment B, average values ± S.D. are shown.

‡ Substrate was human HMW kininogen; individual values are in parentheses. The available amount of the substrate limited the number of bioassays.

§ Not determined.

Table 2. Effect of cycloheximide on amidolytic activity and albumin synthesis rates by the perfused rat liver*

Pretreatment	Amidilytic activity [mU · hr^{-1} · (g liver)$^{-1}$)]	Albumin synthesis [μg · hr^{-1} · (g liver)$^{-1}$]
None (N = 5)	3.8 ± 2.1	209 ± 41
Cycloheximide (N = 5)	0.5 ± 0.2†	138 ± 14‡

* Amidolytic activity was measured with Pro-Phe-ArgpNA. The activities found at 2 min were used as blanks. Rat liver was exsanguinated and perfused with oxygenated Tyrode's solution containing bovine serum albumin (40 mg/ml) at a flow rate of 12–16 ml/min. Cycloheximide treatment consisted of 2 mg/kg, i.p., 17 hr before perfusion, plus 3 mg/kg, i.p., 2 hr before perfusion; 50 μg/ml was added in the perfusion fluid.

† Significantly different from control ($P < 0.01$).

‡ Significantly different from control ($P < 0.05$).

time. As both plasmin and plasma kallikrein are enzymes capable of splitting TAME, in two perfusion experiments amidolytic activity was determined using Val-Leu-LyspNA, a substrate that is seven times more sensitive to plasmin than to plasma kallikrein [15], and Pro-Phe-ArgpNA, a relatively specific substrate for plasma kallikrein [16]. As shown in Table 1 (Expt. A), the amidolytic activity with the substrate for plasmin remained essentially constant. With the substrate for plasma kallikrein the values increased with time.

It could also be shown (Table 1, Expt. B) that the values obtained with the substrate for plasma kallikrein correlated well with the kinin-releasing activity of the perfusates when human HMW kininogen was used as substrate. It was calculated from the values at 2 min and at 120 min that the kinin-releasing activity accumulated in the perfusates at an average rate of 4.2 units · hr^{-1} · (g liver),$^{-1}$ while that for the plasma kallikrein amidolytic substrate was 3.8 ± 2.1 mU·hr^{-1}·(g liver)$^{-1}$.

Effect of cycloheximide on the synthesis rates. As shown in Table 2, pretreatment of the rats with cycloheximide, a known protein synthesis inhibitor, reduced the synthesis rate of the amidolytic activity as measured with the substrate for plasma kallikrein from 3.8 ± 2.1 to 0.5 ± 0.2 mU · hr^{-1} · (g liver)$^{-1}$. Albumin synthesis was also reduced from 209 ± 41 to 138 ± 14 μg·hr^{-1}·(g liver)$^{-1}$.

Table 3. Synthesis of prekallikrein by perfused rat liver*

Expt.	Amidolytic activity [mU · hr^{-1} · (g liver)$^{-1}$]	
	Before activation by HFa	After activation by HFa
1	0.9	3.7
2	2.8	6.2
3	2.4	7.1
4	2.8	6.8
5	< 0.1	9.4
Average ± S.D.	1.8 ± 1.3	6.6 ± 2.0

* Aliquots (0.1 ml perfusate) were collected at 0, 2, 60 and 120 min of perfusion and incubated for 2 min at room temperature with 0.1 ml of 0.04 M Tris–HCl buffer, pH 8.0, in the absence or presence of 30 μl of active Hageman factor (HFa); the final volume was 0.3 ml. *N*-Benzoyl-Pro-Phe-ArgpNA was used as substrate. This amidolytic substrate is 3-fold less sensitive to plasma kallikrein than is Pro-Phe-ArgpNA [15]. The synthesis rates were calculated from the values found.

Synthesis of prekallikrein by rat liver. The kallikrein activity found in the perfusates was probably generated from prekallikrein newly synthesized by the liver. Attempts were made to activate this prekallikrein by three different activation methods: (1) incubation with HFa, (2) acid treatment, and (3) addition of dextran sulfate. Neither acid activation nor the addition of dextran sulfate was capable of increasing the amidolytic activity in these perfusates. As shown in Table 3, however, rat plasma HFa could readily increase this activity from 1.8 ± 1.3 to 6.6 ± 2.0 mU·hr^{-1}·(g liver)$^{-1}$. The amount of active enzyme found in these perfusates (before activation by HFa) varied considerably with the individual rat [< 0.1 to 2.8 mU·hr^{-1}·(g liver)$^{-1}$]. Much less variation was encountered when both precursor and active enzyme (after activation by HFa) were measured [3.7 to 9.4 mU·hr^{-1}·(g liver)$^{-1}$].

Similar experiments using the substrate for plasmin (Val-Leu-LyspNA) and streptokinase as activator gave no increase in amidolytic activity over that found in the absence of streptokinase. A 1:10 dilution of rat plasma in the presence of streptokinase contained 4.5 mU/ml.

Failure of rat liver to activate prekallikrein. As pure rat plasma prekallikrein was not available, human prekallikrein [11] was added to the perfusion fluid at a concentration approximately 7% of that found in normal human plasma; no activation of the prekallikrein occurred during 60 min of liver perfusion. As shown in Table 3, however, 27 per cent (average) of the total rat plasma kallikrein activity was already present in the perfusates before complete activation was obtained by HFa. We observed, further, that diluted (1:10) rat plasma (freshly separated from citrated blood and diluted with calcium-free Tyrode's solution containing 15 mg/ml bovine albumin), which was recirculated through the perfusion system without the liver in it, had activated about 10 per cent of its prekallikrein by the end of 60 min of perfusion; the inclusion of the liver in the system did not modify the plasma prekallikrein activation by the system itself. No activation occurred when 1:10 diluted rat plasma was merely kept at 37° for 60 min.

Clearance of rat plasma kallikrein and human plas-

Table 4. Clearances of rat plasma kallikrein and human plasmin from rat liver perfusates*

	Rat plasma kallikrein	Human plasmin
Perfusion time (min)	Clearance rates (amidolytic activity with Pro-Phe-ArgpNA) (N = 3)	Clearance rates (amidolytic activity with Val-Leu-LyspNA) (N = 1)
2	5.0	3.6
30	0.61	0.72
60	0.34	0.46
120	0.22	0.32

* Clearance rates are expressed as $mU \cdot min^{-1} \cdot (g\ liver)^{-1}$. Initial concentrations in the perfusate were 16.2 mU/ml for plasma kallikrein and 19.5 mU/ml for plasmin.

min by the liver. The possibility was investigated that the liver might not only synthesize prekallikrein but might also inactivate active kallikrein. When either rat plasma kallikrein or human plasmin was added to the perfusion fluid, it was verified that these enzymes were removed and/or inactivated by the liver. Amidolytic activities decreased progressively, reaching about 30 per cent of the initial values after 2 hr of recirculation through the perfused liver. A rapid clearance rate was observed in the first 2 min succeeded by a slow phase that followed an exponential curve (Table 4). In perfusions without the liver, no inactivation of the enzymes occurred.

When human plasma kallikrein [11] was added to the perfusion fluid at a concentration approximately 7% of that found in normal human plasma (six experiments), as with rat plasma kallikrein, only 45 per cent was recovered after 60 min of perfusion. On the other hand, when rat urinary kallikrein (1.8 mU/ml) (in two experiments) was perfused through the liver, no inactivation occurred after 120 min of perfusion. In these latter experiments, amidolytic activity was measured with Val-Leu-ArgpNA, a substrate for urinary kallikrein. Control experiments showed that, in the absence of rat urinary kallikrein, the 2-hr perfusate contained 0.4 mU/ml, measured with Val-Leu-ArgpNA.

DISCUSSION

These studies show that the exsanguinated and perfused rat liver is capable of synthesizing prekallikrein, a finding that constitutes the first direct evidence that plasma prekallikrein is synthesized in this organ. Indeed, following its partial activation in the perfusion system the enzyme activity that accumulated in the perfusates liberated a kinin from pure human HMW kininogen at a rate which paralleled that at which it hydrolyzed its synthetic chromogenic substrate (Pro-Phe-ArgpNA). Furthermore, its activation by HFa, a specific activator of prekallikrein [cf. Ref. 18], supports the bioassay data which indicate that plasma kallikrein, and no other kininogenase was the kinin-generating enzyme. The perfusates also hydrolyzed TAME and had a negligible effect on Val-Leu-LyspNA, a plasmin substrate. Pretreatment of the rats with the protein synthesis inhibitor cycloheximide produced a clear inhibition of the rate of synthesis of both plasma kallikrein, measured with the synthetic chromogenic substrate, and albumin, measured by immunoassay. In fact, the inhibition of kallikrein synthesis was more striking than that of albumin.

The amount of active enzyme (kallikrein) found in the liver perfusates varied widely among the individual rats. In five liver perfusates the amount of active kallikrein formed varied from less than 1.2 to 45 per cent (average 27 per cent) of the prekallikrein that had been synthesized. On the other hand, the total amount of prekallikrein synthesized by these same livers varied only from 3.7 to 9.4 $mU \cdot hr^{-1} \cdot (g\ liver)^{-1}$. Human prekallikrein was not activated during perfusion of rat livers. When diluted (1:10) rat plasma was recirculated for 60 min in the perfusion system, however, 10 per cent of its prekallikrein was activated even when the liver was excluded and despite the fact that all of the equipment had been suitably treated to inactivate negative surfaces. When the diluted rat plasma was not recirculated in the perfusion system, no activation occurred, suggesting that some component in the oxygenating system may have been responsible. In any event, these observations throw serious doubt on the ability of the liver to induce activation of prekallikrein or other early components of the intrinsic coagulation system. Certainly, however, the activation of prekallikrein in these perfusates can explain the loss of kininogen in our earlier studies [8].

We observed that, following full activation of prekallikrein, the perfused rat liver synthesized $6.6 \pm 2.0\ mU \cdot hr^{-1} \cdot (g\ liver)^{-1}$ of kallikrein when measured with benzoyl-Pro-Phe-ArgpNA, which is equivalent to about $20\ mU \cdot hr^{-1} \cdot (g\ liver)^{-1}$ if measured with Pro-Phe-ArgpNA—a substrate 3-fold more sensitive to plasma kallikrein [15]. Using these figures within 30 hr one liver would be able to synthesize 50 per cent of the prekallikrein content of rat plasma. In addition, the rate of albumin synthesis by these rats was, on the average, $209\ \mu g \cdot hr^{-1} \cdot (g\ liver)^{-1}$, which is almost twice that which we found previously [8] and similar to the rate of synthesis [$280\ \mu g \cdot hr^{-1} \cdot (g\ liver)^{-1}$] observed by Hoffenberg *et al.* [19], who perfused rat livers with rabbit blood.

Although the liver was clearly capable of synthesizing plasma prekallikrein, no evidence could be found that it was capable of synthesizing plasmin

and/or plasminogen. The liver perfusates contained only small amounts of an enzyme capable of digesting Val-Leu-LyspNA, an amidolytic substrate which is seven times more sensitive to plasmin than to plasma kallikrein [15]. Further, this enzyme activity did not increase as the perfusion time was prolonged. In addition, streptokinase (a plasminogen activator) did not increase amidolytic activity over that found in the absence of streptokinase. Indeed, Highsmith and Kline [20] reported that, in cats, the kidney is the primary source of plasminogen.

The liver, in addition to synthesizing prekallikrein, also rapidly inactivated rat and human plasma kallikreins and human plasmin, but not rat urinary kallikrein. The clearances were biphasic, showing an initial rapid phase (within 2 min) followed by a slower one, and is similar to the removal of activated factor X by perfused rabbit liver [21]. The metabolism of these enzymes by the liver, however, was obviously much slower than its ability to inactivate bradykinin [13].

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