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Initiation of plasma prorenin activation by Hageman factor-dependent conversion of plasma prekallikrein to kallikrein*

(renin-angiotensin/plasmin/urinary kallikrein/coagulation-kinin)

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ABSTRACT **Plasma prorenin is an inactive form of renin (EC 3.4.99.19) that can be converted to active renin in acid-treated plasma by an endogenous serine protease that is active at alkaline pH (alkaline phase activation). To identify this enzyme we first tested the ability of Hageman factor fragments, plasma kallikrein (EC 3.4.21.8), and plasmin (EC 3.4.21.7) to activate prorenin in acid-treated plasma. All three enzymes initiated prorenin activation; 50% activation was achieved with Hageman factor fragments at 1 μg/ml, plasma kallikrein at 2–4 μg/ml, or plasmin at 5–10 μg/ml. We then showed that the alkaline phase of acid activation occurred normally in plasminogen-free plasma but was almost completely absent in plasmas deficient in either Hageman factor or prekallikrein; alkaline phase activation was restored to these latter plasmas when equal parts were mixed together. Therefore, both Hageman factor and prekallikrein were required for alkaline phase activation to occur. We then found that, although plasma kallikrein could activate prorenin in plasma deficient in either Hageman factor or prekallikrein, Hageman factor fragments were unable to activate prorenin in prekallikrein-deficient plasma. These studies demonstrate that alkaline phase prorenin activation is initiated by Hageman factor-dependent conversion of prekallikrein to kallikrein which, in turn, leads to activation of prorenin. In this fashion, we have revealed a possible link between the coagulation–kinin pathway and the renin–angiotensin system.**

Prorenin is an inactive form of renin (EC 3.4.99.19) that circulates in human plasma. It can be maximally activated by incubation with trypsin (EC 3.4.21.4) (3) to yield a level of renin that is approximately five times the endogenous active renin level (4). Although there is no evidence that plasma prorenin is normally activated *in vivo*, it can be activated in plasma *in vitro* by acidification (5, 6) or incubation at −4°C (cryoactivation) (7, 8). We have shown that only up to one-third of the plasma prorenin that can be activated by trypsin is generated during acidification to pH 3.3, and that further activation does not proceed until a second incubation is carried out at pH 7.4 (9). Both cryoactivation and the alkaline phase of "acid" activation are mediated by neutral serine proteases (9, 10). The purpose of the present study was to identify the plasma proteases responsible for the alkaline phase of acid activation of prorenin.

We have already shown that plasma prorenin can be activated *in vitro* by urinary kallikrein (EC 3.4.21.8) (11). This renal enzyme, however, is unlikely to be responsible for alkaline phase activation in plasma because its plasma concentration is low and it is not inhibited by soybean trypsin inhibitor (12), a substance that completely inhibits alkaline phase activation (10). We therefore investigated whether the neutral serine proteases of the coagulation or fibrinolytic systems could be involved.

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In this report we demonstrate that the alkaline phase of prorenin activation is initiated by Hageman factor-dependent conversion of plasma prekallikrein to kallikrein. Plasma kallikrein, in turn, leads to activation of prorenin.

MATERIALS AND METHODS

Source of Plasmas. Plasmas deficient in Hageman factor (Factor XII-deficient plasma), prekallikrein (Fletcher trait plasma), and high molecular weight (HMW)-kininogen (Fitzgerald trait plasma) were collected in 10% sodium citrate and were obtained from George King Biomedicals, Overland Park, KS. A second bleeding of Fitzgerald trait plasma was a gift from Oscar Carretero (Henry Ford Hospital, Detroit, MI). Williams trait plasma, known to be deficient in both high and low molecular weight kininogens (13), was a gift from Robert Colman (Temple University, Philadelphia, PA). Control plasmas, obtained from 18 hypertensive patients, were collected into K_2EDTA. These plasmas were divided into three groups, each containing plasma from six patients, with mean plasma renin activities of 0.75, 2.5, and 7.5 ng of angiotensin I formed per ml per hr, respectively. These were designated as low, medium, and high renin pools.

Plasminogen-free plasma was prepared by twice passing normal citrated plasma over lysine-Sepharose (14). Buffers utilized throughout were made 10 mM in benzamidine to inhibit activation. The plasminogen-free plasma was concentrated by ultrafiltration to the original volume and then dialyzed against pH 7.4 phosphate-buffered saline. This plasma was over 98% plasminogen-free as assessed by radial immunodiffusion using monospecific antibody to human plasminogen. The IgG level of the processed plasma was within 5% of that of the starting material.

Preparation of Enzymes. Human prekallikrein was purified as described by Mandle and Kaplan (15). The specific activity was 30 units/mg if 1 unit is taken as the quantity of prekallikrein present in pooled normal plasma. None of the prekallikrein was in the active state. It was activated to kallikrein by incubation for 1 hr at 37°C with 1% Hageman factor fragments (HFf) by weight. The HFf was then removed by passage over QAE-Sephadex equilibrated with 0.01 M sodium phosphate buffer, pH 8.0, at 1 mS. Human HFf were prepared by digestion of purified Hageman factor (16) with 5% plasma kallikrein; HFf were then separated from the added kallikrein by passage over SP-Sephadex that was equilibrated in 0.01 M phosphate buffer, pH 6.0, at 1 mS. Human plasminogen was prepared as described by Deutsch and Mertz (14), using lysine-Sepharose affinity chromatography. Plasminogen was eluted from the column with 0.2 M ε-aminocaproic acid and then dialyzed

Abbreviations: HMW-kininogen, high molecular weight kininogen; HFf, Hageman factor fragments.

* Published in preliminary form in refs. 1 and 2.

against phosphate-buffered saline. On the day of the study plasminogen was converted to plasmin (EC 3.4.21.7) by incubation for 1 hr at 30°C with streptokinase (550 international units/mg of plasminogen). Human urinary kallikrein was a gift from Narendra Oza and James W. Ryan of Miami, FL (17).

Acid Activation of Prorenin. *Acid phase.* A 0.5-ml sample of plasma was dialyzed at 4°C to pH 3.3 for 24 hr (0.05 M glycine·HCl/0.1 M NaCl/3 mM Na_2EDTA) and then to pH 5.7 for 24 hr (0.05 M sodium maleate/0.10 M NaCl/3 mM Na_2EDTA). We have previously shown that the acid phase of prorenin activation goes to completion during pH 3.3 dialysis and no further activation occurs during dialysis to 5.7 (9).

Alkaline phase. For the combined acid and alkaline phases, 0.5 ml of plasma was dialyzed at 4°C for 24 hr to pH 3.3 (acid phase) then for 24 hr to pH 7.4 (0.1 M sodium phosphate/0.05 M NaCl/3 mM Na_2EDTA). The plasma was then adjusted to pH 5.7 with 0.276 M maleic acid for renin assay (18). The alkaline phase of activation was calculated from the renin activity after pH 3.3/7.4 dialysis minus the renin activity after pH 3.3/5.7 dialysis.

Trypsin Activation of Prorenin. A 0.3-ml sample of plasma was placed at −4°C for 1 hr; 3 μl of trypsin [Boehringer Mannheim, specific activity 33 benzoyl-L-arginine ethyl ester (BAEE) units/mg] at 100 mg/ml was added and the mixture was incubated for 1 hr at −4°C. Then 1.5 μl of 5% phenylmethylsulfonyl fluoride in ethanol was added and the pH was titrated to 5.7 with 0.276 M maleic acid (4).

Preparation of Acid-Treated Plasma. Fifty milliliters of pooled human plasma in 3 mM K_2EDTA was dialyzed to pH 3.3 for 24 hr at 4°C (0.05 M glycine·HCl/0.1 M NaCl/3 mM Na_2EDTA). This pool was then divided into 4-ml aliquots and stored at −40°C. On the day of the study the acidified plasma was adjusted to pH 7.4 at 0°C with 0.1 vol of 4 M Tris base and approximately 0.01 vol of 2 M NaOH. The plasma was warmed to 25°C prior to study, causing the pH to fall to 7.0.

Under these conditions, the alkaline phase of activation is slow in onset, with a lag time at 25°C of close to 6 hr. This lag time was used to test the effects of various enzymes on the activation of prorenin.

Enzymatic Conversion of Prorenin to Renin. Acid-treated plasma (0.5 ml) was preincubated for 1 hr at 25°C and pH 7.0. Enzymes or buffer control were added in volumes ranging from 5 to 40 μl and the mixture was further incubated for 1 hr at 25°C. The reaction was stopped by placing the samples on ice, adjusting the pH to 5.7 and adding phenylmethylsulfonyl fluoride, as described above for trypsin activation. The renin content was then assayed as described below. Because plasma renin substrate had been destroyed during the acidification step (9), the enzymatic activation studies were carried out in the absence of renin substrate.

Renin Assay. Renin was measured from the rate of angiotensin I formation during incubation at 37°C and pH 5.7 in the presence of 3 mM Na_2EDTA/1. 5 mM phenylmethylsulfonyl fluoride/0.1% neomycin sulfate (18). The angiotensin I formed was quantitated by radioimmunoassay. A small amount of immunoreactive material is always present in plasma prior to incubation for renin assay, therefore this was also measured and subtracted from that measured after 37°C incubation. Because pH 3.3 dialysis destroyed approximately 90% of the endogenous renin substrate, highly purified homologous substrate (19) was added to previously acidified plasmas at pH 5.7 to restore the original substrate concentration before renin assay. Renin substrate was measured as the amount of angiotensin I found after 1-hr incubation of 20 μl of plasma in the presence of excess semipurified renal renin (0.066 Goldblatt unit/ml) prepared according to the method of Haas *et al.* (20).

RESULTS

In three pools of control plasma with low, medium, or high plasma renin activity (Table 1) we confirmed that, normally, close to 20% of the prorenin activable by trypsin is converted to renin during acidification, whereas between 61% and 78% is activated during the alkaline phase. Together the acid and alkaline phases account for 80–97% of trypsin-activable prorenin. Similar degrees of acid and alkaline activation were observed in plasmas deficient in Factors XI, IX, VIII, X, and VII (not shown). The acid phase of activation was also normal in both sources of plasma deficient in HMW-kininogen (Fitzgerald and Williams plasmas), but in each case the alkaline phase was approximately half normal. In contrast, whereas the acid phase again appeared normal in plasma deficient in Hageman factor or prekallikrein, the alkaline phase of activation was almost completely absent (Table 1). However, alkaline phase activation was restored to these plasmas when equal amounts of both were mixed together. Thus the alkaline phase of activation appeared to be dependent on both Hageman factor and prekallikrein.

The mean and SEM for trypsin-activable prorenin is nor-

Table 1. Degree of activation of prorenin by acid and alkaline phases of acid activation

Plasma*	Renin values, ng angiotensin I/ml per hr†			Renin acitivated, %‡	
	Acid phase	Alkaline phase	Trypsin	Acid phase	Alkaline phase
Control					
Low renin	3.6	11.2	18.3	19	61
Medium renin	4.4	18.5	23.5	19	78
High renin	5.7	18.8	27.8	20	68
Deficient					
Hageman factor (n = 3)	2.7	0.4	10.9	25	3
Prekallikrein (n = 4)	7.7	2.2	32.3	24	7
1:1 mixture of Hageman and prekallikrein (n =1)	4.0	10.5	18.7	20	53
Williams trait (n = 2)	5.6	10.0	27.9	20	36
Fitzgerald trait (n = 3)	2.1	2.3	7.2	29	32

* n, Number of plasmas assayed.
† Endogenous renin, prior to activation, has been subtracted.
‡ Expressed as percent of renin released by trypsin activation.

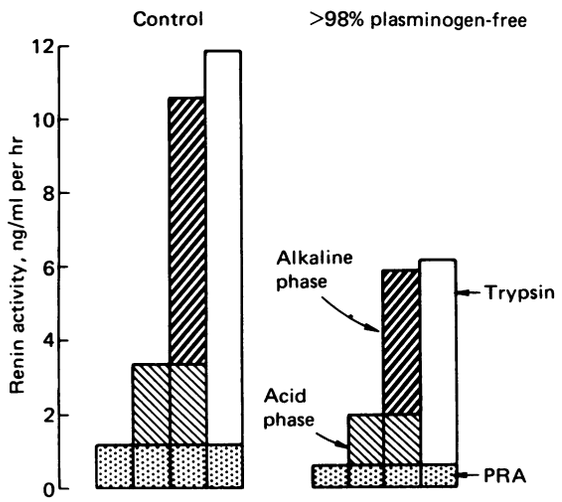

FIG. 1. The alkaline phase of prorenin activation occurs normally in plasma rendered >98% plasminogen-free by passage over lysine-Sepharose. Removal of plasminogen resulted in proportional reductions in both active and inactive renin, but the degree of activation by the acid and alkaline phases, relative to trypsin activation, was unchanged. PRA, plasma renin activity.

mally 13.4 ± 1.9 ng/ml per hr (range 5.7–28.1) (4), and it is interesting to note that the prorenin content of Williams plasma was high (27.9 ng/ml per hr), whereas active renin (0.32 ± 0.11 ng/ml per hr) and prorenin (7.2 ± 0.7 ng/ml per hr) were relatively low in Fitzgerald plasma. The prorenin content of prekallikrein-deficient plasma was also unusually high, whereas the prorenin content of Hageman factor-deficient plasma was within normal limits.

When normal plasma was made over 98% plasminogen-free by two passages over lysine-Sepharose, close to 50% of both active renin and prorenin were lost (Fig. 1). These losses may have been due to nonspecific binding to Sepharose or, alternatively, instability of prorenin. However, the percent activation observed in both the acid and alkaline phase was normal (24% and 71%, respectively) (Fig. 1). Thus both the acid and alkaline phase of prorenin activation appeared to be independent of plasminogen.

The results shown in Table 1 and Fig. 1 suggest that Hageman factor and prekallikrein (but not plasminogen) are required for the alkaline phase of prorenin activation in plasma. Because activated Hageman factor can convert prekallikrein to kallikrein and kallikrein can, in turn, activate Hageman factor (16) and because both initiate activation of the intrinsic coagulation and fibrinolytic pathways (21–24), we next compared the abilities of purified activated Hageman factor (HFf), plasma kallikrein, and plasmin to activate prorenin in normal plasma. We further compared the activation observed with that obtained with urinary kallikrein. As shown in Fig. 2, HFf were potent initiators of prorenin activation, yielding over 50% prorenin activation at 1–2 μg/ml. Plasma kallikrein and urinary kallikrein also readily activated prorenin and were approximately equipotent, producing 50% activation at 2–4 μg/ml, while added plasmin was somewhat less active.

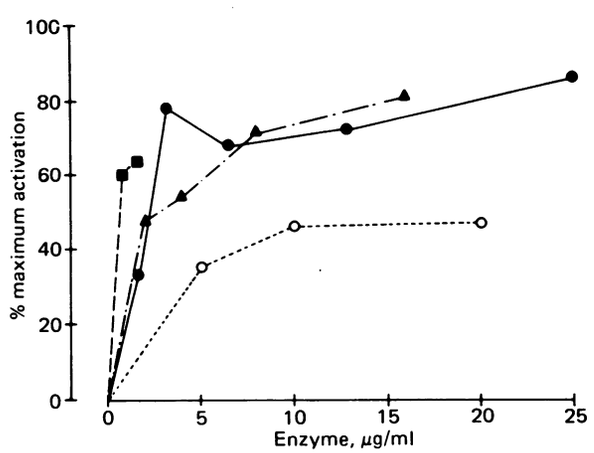

FIG. 2. Activation of plasma prorenin in previously acidified plasma by plasma kallikrein (▲), HFf (■), plasmin (○), and urinary kallikrein (●). Incubation was at pH 7.4 for 1 hr at 25°C. Maximum activation (taken as 100%) was evaluated after incubation of whole plasma with trypsin at 1 mg/ml for 1 hr at −4°C.

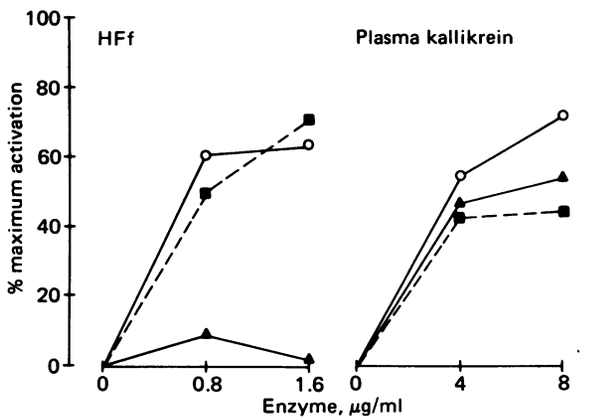

FIG. 3. Activation of prorenin by HFf (*Left*) and plasma kallikrein (*Right*) in control plasma (○) and in plasmas deficient in these enzymes (■, HFf; ▲, prekallikrein). The failure of HFf to activate prorenin in prekallikrein-deficient plasma indicates that HFf activation of prorenin is mediated via plasma kallikrein.

The ability of plasmin to function as an activator of plasma prorenin was confirmed by activating the endogenous plasminogen in the plasma with streptokinase. A streptokinase concentration of 80 units/ml led to 60% prorenin activation. Activation of plasma prorenin by plasmin was not dependent on the presence of prekallikrein, because plasmin at 10 μg/ml led to 75% prorenin activation in prekallikrein-deficient plasma.

To determine whether activated Hageman factor or plasma kallikrein was more directly responsible for alkaline phase activation of prorenin, we next tested the ability of HFf and kallikrein to convert prorenin in plasma deficient in Hageman factor or prekallikrein. As shown in Fig. 3 *left*, when HFf were incubated with normal plasma a concentration of 0.8 μg/ml activated over 50% of the prorenin. A similar degree of activation was obtained when HFf were incubated with Hageman factor-deficient plasma. In contrast, HFf did not lead to significant conversion of prorenin to renin in prekallikrein-deficient plasma. This result suggested that conversion of prorenin to renin by activated Hageman factor was dependent upon the presence of prekallikrein. We therefore tested the ability of purified plasma kallikrein to activate prorenin. As shown in Fig. 3 *right*, addition of kallikrein to normal, Hageman factor-deficient, or prekallikrein-deficient plasmas resulted in comparable activation of prorenin.

DISCUSSION

Previous research has revealed that urinary kallikrein can promote prorenin activation (11), and a recent report by Inagami *et al.* (25) suggests that plasma kallikrein can act similarly. Because plasma kallikrein is a critical component of Hageman

factor-dependent pathways of coagulation, fibrinolysis, and kinin formation, we have evaluated the contribution of these pathways to the alkaline phase of prorenin activation. The data in Table 1 indicate that the alkaline phase of prorenin activation is virtually absent in Hageman factor-deficient and prekallikrein-deficient plasmas. The correction of this abnormality observed after mixing those plasmas indicates that both Hageman factor and prekallikrein contribute to the alkaline phase of prorenin activation.

In examining the question of which enzyme is more directly responsible for prorenin activation, we observed that, although HFf (a form of activated Hageman factor) and plasma kallikrein each led to prorenin activation in normal plasma and in Hageman factor-deficient plasma, HFf did not activate prorenin in prekallikrein-deficient plasma. This finding indicates that activated Hageman factor does not activate prorenin directly but does so by first converting prekallikrein to kallikrein.

Because kallikrein has been reported to activate plasminogen (15, 26), it was possible that the observed activation of prorenin is a plasmin-dependent phenomenon. We, therefore, investigated the ability of plasmin (streptokinase-activated plasminogen) to activate prorenin. Our data demonstrate that plasmin is indeed capable of activating prorenin, a finding in agreement with observations of Osmond *et al.* (27). However, we believe it unlikely that plasmin mediates the Hageman factor-dependent prorenin activation because: (*i*) kallikrein is an exceedingly weak activator of plasminogen when compared to streptokinase or urokinase (unpublished observations) and (*ii*) plasma that has been rendered over 98% plasminogen-free has a normal alkaline phase of prorenin activation (Fig. 1). It therefore appears likely that kallikrein either directly converts prorenin to renin or functions via activation of other proenzymes that are as yet unidentified. Studies utilizing purified prorenin are required to definitively resolve these possibilities.

HMW-kininogen, a required cofactor for contact activation of prekallikrein and Hageman factor (13, 28, 29), augments both activation of prekallikrein by activated Hageman factor and activation of Hageman factor by kallikrein (30, 31). Although the degree of alkaline activation of prorenin was subnormal in two plasmas deficient in HMW-kininogen, it was reduced by only 50% (Table 1), in contrast to the 90% depression observed in contact activation of prekallikrein (13). Because the activation of prorenin during the acid and alkaline phases appears to be predominantly a fluid phase, rather than a surface-dependent phenomenon, it is possible that HMW-kininogen plays a less significant role in prorenin activation. This is consistent with the observation that HMW-kininogen functions as a cofactor by binding to prekallikrein and coagulation Factor XI (32, 33) and facilitates their attachment to negatively charged surfaces (34). Thus, in a fluid phase system the contribution of HMW-kininogen to prekallikrein activation is substantially less than that observed during contact activation (35). In the present studies we utilized HFf as the activated form of Hageman factor because HFf does not bind to surfaces (36) and is an effective fluid phase activator of prekallikrein (16).

Cryoactivation of plasma prorenin is also mediated by neutral serine proteases (9, 10). Osmond and coworkers have reported that, although cryoactivation does not occur normally in Hageman factor-deficient plasma (37), it does occur in prekallikrein-deficient plasma (27). Our own results (unpublished observations) indicate that cryoactivation is subnormal in both deficient plasmas but, unlike the acid–alkaline activation process in the experiments described herein, mixtures of these deficient plasmas also do not cryoactivate normally. In contrast to our own results, Millar *et al.* (38) have reported that "acid" activation of prorenin in prekallikrein-deficient plasma is close to normal, but they did not analyze separately the acid and the alkaline phases, nor did they quantitate the total activable prorenin (which is unusually high in this plasma). Thus the observed activation was undoubtedly partial and attributable only to the acid phase.

In normal plasma a reciprocal reaction involving Hageman factor and prekallikrein occurs such that activated Hageman factor converts prekallikrein to kallikrein and kallikrein enzymatically activates more Hageman factor. This would lead to a rapid accumulation of both kallikrein and activated Hageman factor (HFf) in plasma were it not for the presence of plasma inhibitors that inactivate these enzymes (see Fig. 4). After acid treatment of plasma many plasma inhibitors are

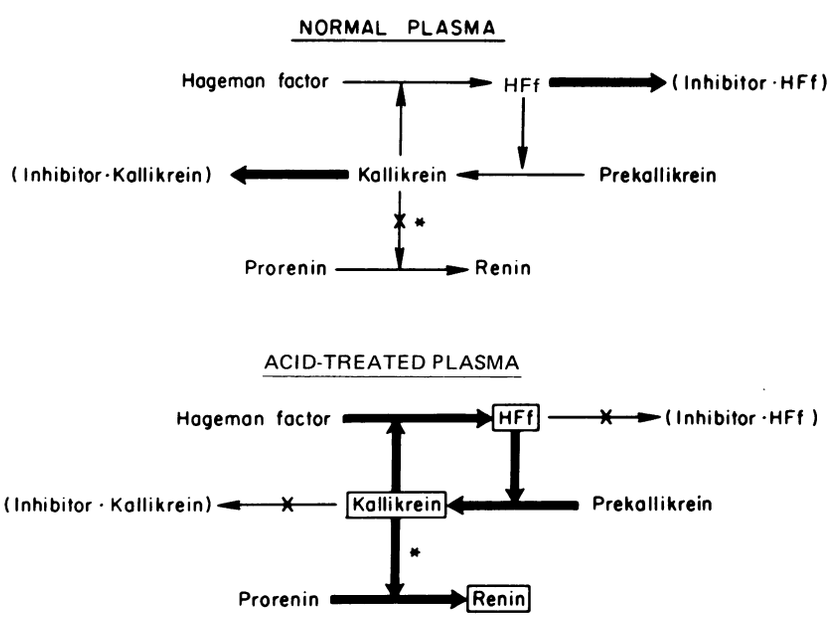

FIG. 4. Proposed cascade by which Hageman factor-dependent activation of prorenin occurs in acid-treated plasma and normal plasma. Heavy arrows indicate predominant pathway in each instance.
* Kallikrein activation of prorenin may be direct or indirect.

destroyed (39, 40) so that, as reciprocal activation of Hageman factor and prekallikrein proceeds, the concentration of active kallikrein becomes high enough to initiate conversion of prorenin to renin. Further studies are needed to evaluate whether activation of the renin-angiotensin cascade can occur in untreated plasma, to compare the contact and fluid phase mechanisms for the generation of bradykinin and renin, and to evaluate whether plasma kallikrein activates prorenin directly or via activation of an as yet unidentified proenzyme. Meanwhile, this research further defines the alkaline phase of prorenin activation and suggests a possible link between the coagulation–kinin and the renin–angiotensin systems.

Note Added in Proof. After submission of this manuscript Derkx *et al.* reported similar results and conclusions (41).

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