Activation of inactive plasma renin by plasma and tissue kallikreins


Department of Internal Medicine I, University Hospital Dijkzigt, Erasmus University, Rotterdam, The Netherlands

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Summary

1. Normal human plasma contains a pro-activator of inactive renin. The pro-activator is activated at physiological pH in plasma that has been pretreated with acid. This activation in vitro leads to the conversion of inactive renin into the active form with simultaneous generation of kallikrein activity.

2. The endogenous activator of inactive renin has the same pH profile and inhibitor spectrum as plasma kallikrein.

3. Inactive renin can also be activated by exposure of plasma to exogenous trypsin, and in normal plasma the quantities of inactive renin that are activated after acidification and with trypsin are identical. Prekallikrein (Fletcher factor)-deficient plasma, however, has much lower renin activity after acidification than with trypsin. Thus acid activation of inactive renin depends on plasma prekallikrein, whereas the action of trypsin is independent of prekallikrein.

4. Highly purified tissue (pancreatic) kallikrein, in a concentration of less than $2 \times 10^{-8}$ mol/l, activates inactive renin that has been isolated from plasma by ion-exchange chromatography. In this respect it is at least 100 times more potent than trypsin.

5. It is therefore possible that plasma and/or tissue (renal) kallikreins are also involved in the activation of inactive renin in vivo.

Key words: kallikrein, kallikrein inhibitor, renin.

Abbreviation: PPAN, \(\text{D-propyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride}\).

Introduction

Plasma and tissue kallikreins (EC 3.4.21.8) act on kininogens in plasma to form bradykinin and kallidin respectively. The preferred substrate for plasma kallikrein is high-molecular-weight kininogen, whereas the tissue kallikreins react more readily with low-molecular-weight kininogen (Erdős, 1976). Furthermore, the formation of kallikrein is an early step in the cascade-like activation of proteolytic plasma factors involved in coagulation, fibrinolysis and complement-mediated reactions (Ogston & Bennett, 1978). Here we report evidence that kallikreins are also capable of activating inactive renin (EC 3.4.99.15).

Methods

Collection of blood

Blood from 20 healthy male subjects was collected in plastic tubes, which contained 5 mmol of edetic acid (EDTA)/l of blood. Within 5 min the blood was centrifuged at 8000 g for 10 min. The plasma was pooled and immediately frozen at $-20^\circ\text{C}$. Plasma was also obtained from a patient with prekallikrein (Fletcher factor)-deficiency (less than 1% of normal).
Activation and assay of renin

Activation and assay of renin by acid. Samples (2 ml) of EDTA-treated plasma, or fractions, isolated from plasma by ion-exchange chromatography, were dialysed for 24 h at 4°C against a glycine/HCl buffer, pH 3.3, which contained EDTA (5 mmol/l) (Skinner, 1967; Derkx, Wenting, Man in 't Veld, Verhoeven & Schalekamp, 1978a). After the pH had rapidly been restored to 7.5 with NaOH (1.0 mol/l), the samples were dialysed for another 24 h at 4°C against sodium phosphate buffer, pH 7.5, which contained EDTA (1 mmol/l). The pH-dependency of renin activation was assessed by adjusting the pH after the first dialysis step to values ranging from 4.0 to 9.5, again with NaOH (1.0 mol/l), followed by dialysis against citric acid/phosphate buffers of the same pH. In experiments where the rate of activation was followed, the second dialysis step was omitted, and the samples were kept in plastic tubes for various times at 4°C as indicated, after pH had been adjusted to 7.5.

Activation of inactive renin by trypsin and kallikrein. Trypsin (EC 3.4.11.4), 12 000 kallikrein units/mg of protein, was obtained from Sigma Chemical Co. Ltd, St Louis, U.S.A. Highly purified porcine pancreatic kallikrein (EC 3.4.21.8) was kindly supplied by Professor G. L. Haberland and Dr E. Wischhöfer (Kallikrein KZC 45/32, Bayer, Leverkusen, West Germany). The specific activity of this preparation was 1180 kallikrein units/mg of protein. The enzymes were covalently bound to CNBr-activated Sepharose-4B (Pharmacia) in a ratio of 10–30 mg of protein/g of dry Sepharose (Pharmacia, 1974). The immobilized enzymes were added to EDTA-treated plasma or to fractions isolated from plasma by ion-exchange chromatography. The suspensions were slowly shaken at 4°C, for various times as indicated, and the enzymes were then removed by centrifugation at 8000 g for 10 min.

Activation of inactive renin by low temperature. Samples (2 ml) of EDTA-treated plasma were kept in plastic tubes for various times at 4°C as indicated.

Inhibition of activation in vitro of inactive renin. Samples (2 ml) of EDTA-treated plasma were dialysed for 24 h at 4°C against glycine/HCl buffer, pH 3.3. The pH was then restored to 7.5 with NaOH (1.0 mol/l), and the samples were redialysed at pH 7.5 in the presence of various protease inhibitors. The following inhibitors were studied: (1) aprotinin (Trasylol, Bayer); (2) soyabean trypsin inhibitor (Sigma); (3) lima-bean trypsin inhibitor (Sigma); (4) trypsin inhibitor from chicken egg-white (ovomucoid, Sigma); (5) benzamidine/HCl (Sigma).

Assay of naturally occurring active renin. The method, which has previously been described (Derkx et al., 1978a), was slightly modified. Briefly, samples (2 ml) of EDTA-treated plasma or fractions, isolated from plasma by ion-exchange chromatography, were dialysed for 2 x 24 h at 4°C against a phosphate buffer, pH 7.5, which contained EDTA (1 mmol/l). Aliquots (0.1 or 0.2 ml) of the dialysed samples were mixed with sheep renin-substrate (concentration 6.1 mmol/l), and the total volume was adjusted to 1.0 ml with phosphate buffer, pH 7.5. After addition of 10 μl of 8-hydroxyquinoline (340 mmol/l), 5 μl of phenylmethylsulphonylfluoride (287 mmol/l) in ethanol and 10 μl of aprotinin (10 000 kallikrein-inhibiting units/ml) the mixture was incubated for 3 h at 37°C. The angiotensin I that was generated during incubation with renin substrate was measured by radioimmunoassay, and compared with that generated by standard human kidney renin (MRC standard 68/156). With the protease (angiotensinase) inhibitors we have used, recovery of angiotensin I, which was added to plasma after dialysis, was 98.8 ± 4.9% (n = 15). The recovery of standard renin, which was added to plasma before dialysis, was 97.0 ± 4.8% (n = 15).

Assay of renin activated in vitro. After the samples had been treated with acid, trypsin, kallikrein or low temperature, their renin content was measured as described before.

Assay of kallikrein

The method is based on the amidolytic action of plasma kallikrein on the synthetic chromogenic substrate H-D-propyl-L-phenylalanyl-L-arginine-p-
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nitroanilide dihydrochloride (PPAN), as described by Claeson, Friberger, Knös & Eriksson (1978). Kallikrein was measured in EDTA-treated plasma which had been treated with acid, trypsin or low temperature. Aliquots (10 or 100 µl) of the treated plasma were mixed with the chromogenic substrate (PPAN, Kabi, Stockholm, Sweden; concentration 3.7 Kₐ), and the total volume was adjusted to 1.2 ml with pre-warmed (37°C) Tris buffer (50 mmol/l), pH 7.5. The increase in absorbance at 405 nm was followed for 2 min in a 1 cm semi-microcuvette at 37°C. In experiments where the pH-dependency of kallirein activity was assessed, the Tris buffer, pH 7.5, was replaced by citric acid/phosphate buffers with pH values ranging from 4.0 to 9.5. As mentioned before, these buffers were also used for studies on the pH-dependency of renin activation. The results of both sets of experiments could therefore be compared.

Units for expression of results

Renin concentration is expressed as micro-units of the renin standard (MRC standard 68/156)/ml of plasma (µunits/ml). The concentration of inactive renin is the concentration of renin measured in plasma after dialysis for 24 h at pH 3.3 and subsequent dialysis for 24 h at pH 7.5 (total renin), minus the concentration of renin measured after dialysis for 2 × 24 h at pH 7.5 (active renin). Kallikrein concentration is expressed as units/ml of plasma (units/ml), 1 unit being the quantity of enzyme that hydrolyses 1 µmol of the synthetic substrate PPAN in 1 min at pH 7.5 and 37°C.

Results

Simultaneous activation of inactive renin and pre-kallikrein in plasma

In plasma treated at pH 3.3, the activation of inactive renin occurred after pH had been restored to a value above 5.0. The pH-dependency of this process followed a bell-shaped curve, which was identical with the pH/activity curve of plasma kallikrein (Fig. 1).

Results of various activation procedures, carried out on whole plasma at 4°C, are shown in Fig. 2. In normal plasma (Fig. 2a) both inactive renin and prekallikrein were simultaneously activated by either acid or trypsin treatment. Activation of inactive renin after acidification with subsequent restoration of pH to 7.5 was maximal after 16–24 h. The same maximum was reached by treatment with trypsin at pH 7.5 for 24 h. Treatment for longer periods up to 72 h did not result in any further increase of renin activity.

In contrast with normal plasma the amount of inactive renin that was activated in prekallikrein-deficient plasma after acidification was much smaller than with trypsin (Fig. 2b, lower part). There was no detectable amidolytic action on the chromogenic substrate, PPAN, after acid or trypsin treatment of the deficient plasma (Fig. 2b, upper part), which demonstrates that, for the purpose of our experiments, PPAN has adequate specificity for kallikrein.

When normal plasma was kept at 4°C, without any other form of treatment, the activation of both inactive renin and prekallikrein proceeded more slowly (Fig. 2a). Renin activity was 31 ± 6% above control after 48 h, and rose to 45 ± 3% above control after 72 h. The differences from control were significant (P < 0.01, paired t-test). In prekallikrein-deficient plasma no activation occurred under these circumstances.

Effects of protease inhibitors on the activation of inactive renin in plasma

Table 1 shows the effects of protease inhibitors, which were added to acid-treated plasma after the
FIG. 2. Effects of various procedures on the generation of active renin and kallikrein in normal plasma (M ± SEM, n = 10) and in prekallikrein (Fletcher factor)-deficient plasma. The plasma samples were treated as follows: O, storage at 4°C (cold activation); ●, dialysis at pH 3.3, followed by dialysis at pH 7.5; and △, incubation with trypsin (final concentration approximately 1.2 × 10^−6 mol/l). In normal plasma inactive renin and prekallikrein are simultaneously activated with each procedure. No active renin is formed in deficient plasma after storage at 4°C or at pH 7.5 after dialysis at pH 3.3.

pH had been restored to neutral. The inhibitor/plasma mixtures were kept at 4°C for 24 h. Among the substances tested, aprotinin (Trasylol), soya-bean trypsin inhibitor and benzamidine/HCl had the greatest effect on the activation of inactive renin. The inhibitor spectrum for renin activation was similar to that reported for plasma kallikrein (Vogel & Werle, 1970; Kaplan & Austen, 1975). None of the protease inhibitors interfered with the reaction between active renin and its substrate. This was demonstrated by adding the inhibitors to known amounts of human renin (MRC standard 68/156) before incubation with excess of sheep renin substrate.

**Activation of inactive plasma renin by tissue kallikrein**

Pancreas kallikrein, added to whole plasma and left there for 24 h at 4°C, did not activate inactive renin (Table 1). This contrasts with the findings when a semipurified preparation of inactive plasma renin was used instead of whole plasma. As illustrated in Fig. 3, the elution pattern, obtained after DEAE-Sepharose column chromatography of plasma, showed two peaks of renin activity at sodium concentrations of 60 mmol/l (peak A) and 100 mmol/l (peak B). The renin content of peak B comprised 80–90% of the total quantity of active renin present in whole plasma. Acid treatment doubled the renin content of peak A but had no effect on peak B, indicating that peak A contained inactive renin. After acid treatment, however, only a small proportion of the total quantity of inactive renin present in peak A was activated, whereas addition of pancreatic kallikrein, in a concentration of 12 × 10^−8 mol/l of eluate, had a much larger effect. The quantity of inactive renin that could be activated by kallikrein after ion-exchange chromatography was calculated to be 90–95% of the quantity that could be activated by acid in plasma before chromatography. This indicates that 90–95% of the acid-activatable renin in whole plasma is recovered as kallikrein-activatable renin after chromatography. Kallikrein had no effect on peak B. In Table 1 the quantities of kallikrein and trypsin that were needed for the activation of inactive renin are compared. It can be seen that kallikrein is at least 100 times more potent.
The same plasma pool was used in all experiments. The quantity of renin that was activated in whole plasma after 24 h dialysis at pH 3.3 with subsequent 24 h dialysis at pH 7.5 without serine protease inhibitors was taken as 100%. Acid-activatable renin in the plasma pool was 170 μunits/ml. As stated in the text, 90–95% of the acid-activatable renin in whole plasma was calculated to be recovered as kallikrein-activatable renin after chromatography (concentration of added kallikrein in eluate approximately $12 \times 10^{-8}$ mol/l). The activation of semipurified inactive renin was therefore considered to be complete under these circumstances.

### Table 1. Activation of inactive renin by proteases and effects of protease inhibitors

<table>
<thead>
<tr>
<th>Proteases (1) and protease inhibitors (2)</th>
<th>Prior treatment of plasma</th>
<th>Percentage of renin activated ($M \pm$ SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Kallikrein</td>
<td>None</td>
<td>$&lt;5 \pm 6$</td>
</tr>
<tr>
<td>approx. $12 \times 10^{-8}$ mol/l</td>
<td></td>
<td>$&lt;5 \pm 6$</td>
</tr>
<tr>
<td>approx. $1.2 \times 10^{-8}$ mol/l</td>
<td></td>
<td>$100 \pm 5$</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>Ion-exchange</td>
<td>$72 \pm 4$</td>
</tr>
<tr>
<td>approx. $12 \times 10^{-8}$ mol/l</td>
<td>chromatography</td>
<td></td>
</tr>
<tr>
<td>approx. $1.2 \times 10^{-8}$ mol/l</td>
<td>(peak A)*</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>None</td>
<td>$102 \pm 6$</td>
</tr>
<tr>
<td>approx. $1.2 \times 10^{-8}$ mol/l</td>
<td></td>
<td>$75 \pm 6$</td>
</tr>
<tr>
<td>approx. $0.6 \times 10^{-8}$ mol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>Ion-exchange</td>
<td>$56 \pm 6$</td>
</tr>
<tr>
<td>approx. $1.2 \times 10^{-8}$ mol/l</td>
<td>chromatography</td>
<td></td>
</tr>
<tr>
<td>approx. $0.6 \times 10^{-8}$ mol/l</td>
<td>(peak A)</td>
<td>$35 \pm 4$</td>
</tr>
</tbody>
</table>

(1) Kallikrein and renin

Most of the kallikrein and renin circulating in plasma is inactive. The activation of the kallikrein–kinin system may be linked with the activation of the renin–angiotensin system. The evidence for this is as follows: (1) prekallikrein and inactive renin in normal plasma are simultaneously activated after exposure of plasma to acid and subsequent restoration of pH, (2) the pH-dependency of the endogenous plasma enzyme that activates inactive renin closely parallels the pH/activity profile of kallikrein, (3) after acidification there is little activation of inactive renin in prekallikrein-deficient plasma, (4) substances that are known to inhibit plasma kallikrein, such as aprotinin, soya-bean trypsin inhibitor and benzamidine (Vogel & Werle, 1970; Kaplan & Austen, 1975) interfere with the activation of inactive renin, (5) other protease inhibitors that do not inhibit plasma kallikrein significantly, such as lima-bean trypsin inhibitor and ‘ovomucoid’ (Kaplan & Austen, 1975), have little effect, and (6) highly purified tissue (pancreatic) kallikrein is a potent activator of inactive renin, which has been isolated from plasma by ion-exchange chromatography. Exogenous kallikrein has no effect when added to untreated whole plasma, probably because of the inhibitory action of some plasma proteins (Ogston & Bennett, 1978).

Admittedly, the procedures that activate renin
Fig. 3. Separation of inactive renin from active renin by ion-exchange chromatography of normal plasma, and its activation with pancreatic kallikrein (final concentration approximately $12 \times 10^{-8}$ mol/l) or acid. The absorbance by plasma proteins and the sodium gradient are shown in the upper part of the Figure. Peaks A and B in the lower part of the Figure correspond to inactive and active renin respectively (for explanation, see the text). Fractions from the column were treated as follows: ○, dialysis at pH 7.5 for 2 x 24 h; ●, dialysis at pH 3.3 for 24 h, followed by dialysis at pH 7.5 for 24 h; □, dialysis at pH 7.5 for 24 h with subsequent incubation with kallikrein for 24 h; ■, dialysis at pH 3.3 for 24 h with subsequent restoration of pH to 7.5 and incubation with kallikrein for 24 h. These procedures were carried out at 4°C.

also activate other plasma factors besides prekallikrein, and the pH-optimum curve and inhibitor spectrum of kallikrein are shared by several serine proteases involved in the processes of clotting, fibrinolysis and complement activation. Furthermore although both plasma kallikrein and tissue kallikrein are involved in kinin formation, their substrate specificity is somewhat different (Erdos, 1976). Thus we have no proof that inactive renin is directly activated by kallikreins. On the other hand, generation of kallikrein is an early step in the activation of other serine proteases in plasma (Kaplan & Austen, 1975; Ogston & Bennett, 1978). Furthermore, in prekallikrein-deficient plasma, renin activity after acidification was much smaller than with trypsin, whereas, in normal plasma, the results with acid and trypsin were identical. This is strong evidence that acid activation of inactive renin depends on plasma prekallikrein. Our data therefore suggest that the activation of inactive renin is at least in some way linked with the kallikrein–kinin system. During the course of this work some preliminary data, which are in keeping with this view, have been published (Leckie, 1978; Derkx, Tan-Tjong & Schalekamp, 1978b; Morris & Day, 1978; Sealey, Atlas, Laragh, Oza & Ryan, 1978). Sealey et al. (1978) recently reported that renin activity increased more rapidly when urinary kallikrein, which originates from the kidney (Obika, 1978), was added to acid-treated plasma than with acid treatment alone. Our work extends these observations in that it demonstrates that inactive renin can be fully activated by tissue kallikrein without prior acid treatment.

**Physiological implications**

The physiological importance of these data remains to be established, but some observations might be relevant. Active kallikrein is secreted by kidneys and salivary glands, and these organs also produce active renin (Webster, 1970; Obika, 1978). Furthermore, there is some evidence for intrarenal activation of inactive renin (Derkx, Wenting, Man in 't Veld, Verhoeven & Schalekamp, 1979). Plasma bradykinin and urinary kallikrein increase when the release of active renin is stimulated by standing up or sodium depletion (Wong, Talamo, Williams & Colman, 1975; Margolius, Horwitz, Geller, Alexander, Gill, Pisano & Keiser, 1974; Levy, Frigon & Stone, 1978). Furthermore, some stimuli for plasminogen activation, such as exercise and isoprenaline (Brozovic, 1977), increase the release of active renin (Fasola, Martz & Helmer, 1966; Aurell & Vikgren, 1971; Derkx, Wenting, Man in 't Veld, Van Gool, Verhoeven & Schalekamp, 1976; Fagard, Amery, Reybrouck, Lynen, Moerman, Bogaert & De Schaepdryver, 1977) and may activate prekallikrein (Ogston & Bennett, 1978). Possibly related to this is the frequent association of malignant hypertension with low-grade disseminated intravascular coagulation and high concentrations of circulating active renin (Gavras, Brown, Lever, Linton, MacAdam, MacNicol, Robertson & Wardrop, 1971). In the intact organism, kallikrein-mediated activation of proteolytic enzymes involved in clotting and fibrinolysis is thought to occur in close contact with the blood-vessel wall, and after their release into the circulation they are rapidly inactivated or removed (Ogston & Bennett, 1978).
may also be true for components of the renin–
angiotensin system, so that circulating levels of
these hormones correlate poorly with their physio-
logical effects. Finally, the biologically active end-
products of the kallikrein–kinin and renin–angio-
tensin systems act on vascular smooth muscle.
Kinin are potent vasodilators, and angiotensin II is
a potent vasoconstrictor. A direct interaction of
kallikrein and renin, possibly in close association
with the blood-vessel wall, might therefore provide
a mechanism for blood-pressure regulation.

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