Activation of a Prorenin-Like Substance in Human Plasma by Trypsin and by Urinary Kallikrein

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SUMMARY

Inactive plasma renin (a prorenin-like substance) can be activated by trypsin. There are also endogenous neutral serine proteases in plasma that can activate inactive renin in vitro, but only after protease inhibitors are either destroyed by acidification (the alkaline phase of acid activation) or inactivated by cold (cryoactivation). In the present study we have shown that cold also facilitates trypsin activation. But even at -4°C, as much as 1 mg/ml of trypsin was required to overcome endogenous inhibitors and reproducibly activate inactive plasma renin during a 1-hour incubation at pH 7.4. After partial destruction of plasma protease inhibitors by acidification to pH 3.3, less trypsin was required for complete activation at pH 7.4: 200 μg/ml at 25°C and 100 μg/ml at -4°C. In contrast, 10 μg/ml of the renal enzyme urinary kallikrein completely activated inactive renin in previously acidified plasma at 25°C. Maximum activation of inactive plasma renin by trypsin or renal kallikrein was almost identical. Both enzymes caused activation in plasmas deficient in Hageman factor or Fletcher factor (prekallikrein), suggesting that their ability to activate inactive renin is not mediated by these neutral serine proteases of the intrinsic coagulation system.

Using maximum trypsin activation to define "total" renin, we found that among 22 normal subjects and hypertensive patients there was a direct relationship between the proportion of active renin in plasma (active/total) and the concurrent urinary kallikrein excretion (r = 0.46, p < 0.05). Normotensive white subjects had a higher proportion of active plasma renin than blacks, in whom urinary kallikrein is reported to be low. Altogether, these data suggest that there might be a link between prorenin and renal kallikrein in vivo. Further studies are required to evaluate this possibility and to determine whether prior hydrolysis of inactive renin, for example, by acidification, is required for renal kallikrein to activate inactive renin.

(Hypertension 1: 179-189, 1979)

KEY WORDS: prorenin • trypsin • renal kallikrein • renin • cryoactivation • serine protease • trypsin inhibitors • urinary kallikrein

There is no evidence that inactive renin is normally converted to active renin in human plasma in vivo, but there are neutral proteases in plasma capable of activating inactive renin1-4 which seem to be completely inhibited.4 Cryoactivation and acid activation, two techniques commonly used to activate inactive plasma renin in vitro, appear to work, in part, by rendering ineffective inhibitors of these neutral serine proteases5,4 which then become capable of activating inactive renin at alkaline pH (fig. 1). Thus, cold appears to inactivate reversibly a protease inhibitor5-6 so that activation of inactive renin proceeds only as long as the temperature remains low.6 On the other hand, acid destroys several inhibitors, including α1 antitrypsin7 and α2 macroglobulin8 and, when the pH is subsequently restored to 7.4, enzymatic activation of inactive renin proceeds (fig. 1).1,2,4 In addition, partial activation of inactive renin (about 30%) occurs in plasma.

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during the acidification step to pH 3.3. This acid phase of activation also appears to be mediated by an enzyme.

In the course of investigating which neutral proteases can activate inactive plasma renin, we found that urinary kallikrein is more potent than trypsin. Since urinary kallikrein comes from the kidney, and since its storage site is close to that of renin, it seemed possible that renal kallikrein might be the in vivo converting enzyme of inactive renin.

In the present study we have investigated further the interrelationships between urinary kallikrein and inactive renin. This required a method for activating reproducibly all of the inactive renin in plasma so that the measurement could be used as a reference for other activation procedures. Since cryoactivation activates only a third of the inactive renin in plasma and since acid activation has the disadvantage of destroying renin substrate, which must be restored before assay, we first identified conditions for reproducible activation by trypsin. Although trypsin had been shown to activate inactive renin, conditions for maximal and reproducible activation in plasma have not been defined. We have also compared the degree to which trypsin and highly purified urinary kallikrein can activate inactive plasma renin. Finally, to investigate further any possible link between inactive plasma renin and urinary kallikrein, we measured the rate of excretion of urinary kallikrein in a group of hypertensive patients and normal subjects and related it to the proportion of active renin in the circulation.

**Methods**

**Processing of Blood and Plasma**

Blood was collected into K$_2$ EDTA Vacutainers (Becton-Dickinson, Clarkson, Ontario) and centrifuged at room temperature. Plasma was either stored frozen at $-40^\circ$C or processed immediately. Frozen plasmas were thawed rapidly in front of a fan and all plasmas were processed at room temperature up to the activation step or until the pH was adjusted to 5.7. Subsequently they were chilled in an ice bucket or refrigerator. In this way cryoactivation before measurement of endogenous renin or activation was avoided.

**Measurement of Inactive Renin (Trypsin)**

Trypsin, (Boehringer Mannheim, specific activity 33 U/mg) 100 mg/ml in 0.0025 N HCl, was stored for a maximum of 1 week at 4°C. A concentration of 1 mg/ml plasma was used to activate inactive renin during a 1-hour incubation at $-4^\circ$C (table 1). A shaking, refrigerated, constant-temperature bath containing methanol maintained the temperature at $-4^\circ$C (FTS Recirculating Cooler RC-6-PRLT attached to New Brunswick Aquatherm G86). Preincubation at $-4^\circ$C for 1 hour ensured a stable temperature before addition of trypsin. The reaction was stopped by adjusting to pH 5.7 with 100 $\mu$1 maleic acid (0.276 M maleic anhydride, pH 1.23) and by addition of 5 $\mu$1 phenylmethylsulfonyl fluoride (PMSF; 5% in ethanol). These reagents, together with 10 $\mu$1 of 10% neomycin sulfate, provided optimal conditions for performing the renin incubation step. For both total and endogenous renin measurements an aliquot of plasma was removed before 37°C incubation for measurement of the blank. A 1-hour incubation period was used for the trypsin-activated samples because the renin value after trypsin activation was almost always above 5 ng/ml/hr and this shorter incubation time eliminated the need to dilute the samples before radioimmunoassay. For all other assays a 3-hour incubation was performed and the results expressed as an hourly rate of angiotensin generation.

Inactive renin (trypsin) was calculated from total renin (minus blank) minus endogenous renin (minus blank). Inactive renin (trypsin) in a pool of plasma, activated nine times, was 16.0 ± 1.5 (SD) ng/ml/hr. The coefficient of variation was 9.4%.
Table 1. Measurement of Inactive Renin by Trypsin Activation

<table>
<thead>
<tr>
<th>Total renin (trypsin)</th>
<th>Endogenous renin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml EDTA plasma</td>
<td>1 ml EDTA plasma</td>
</tr>
<tr>
<td>Preincubate 1 hr at -4°C</td>
<td>Add: 10 µl neomycin</td>
</tr>
<tr>
<td>Add 10 µl trypsin (100 mg/ml): mix</td>
<td>5 µl PMSF : mix</td>
</tr>
<tr>
<td>Incubate for 1 hr at -4°C</td>
<td>Place on ice</td>
</tr>
<tr>
<td>Leave 0.5-ml aliquot on ice (blank)</td>
<td>Place on ice</td>
</tr>
<tr>
<td>Incubate remainder for 1 hr at 37°C</td>
<td>Incubate remainder for 3 hrs at 37°C</td>
</tr>
<tr>
<td>Assay Ang I in blank and incubated samples</td>
<td>Assay Ang I in blank and incubated samples</td>
</tr>
</tbody>
</table>

\[
\text{INACTIVE RENIN (trypsin)} = (\text{TOTAL RENIN} - \text{blank}) - (\text{ENDOGENOUS RENIN} - \text{blank})
\]

Measurement of Inactive Renin (Cryo)

Unadjusted ethylenediaminetetraacetic acid (EDTA) plasma (pH 7.9) was incubated at -4°C for 4 days. Renin was then measured as described above and in table 1. To calculate inactive renin (cryo) the endogenous renin (minus blank) was subtracted from the “total” renin (cryo) (minus blank).

Studies to Determine Optimal Conditions for Trypsin Activation of Inactive Renin

A large pool of EDTA plasma with a plasma renin activity (PRA) of 2.3 ng/ml/hr was used for these studies. Trypsin was diluted with 0.0025 N HCl to obtain the various concentrations illustrated in figures 2, 3 and 5. We added 10 µl of each dilution in duplicate to 1-ml aliquots of human plasma that had been preincubated for 1 hour at the appropriate temperature. The samples were then incubated for up to 48 hours at either -4°C, 6°C, 25°C or 37°C. Otherwise the conditions for incubation and assay were the same as described in table 1.

Effect of Temperature on Trypsin Hydrolysis of BAPNA and Inactive Renin

Hydrolysis of BAPNA (benzoyl-d,l-arginine-p-nitroanilide HCl) was carried out by the method of Eriksson. Increasing volumes of trypsin (0.2 mg/ml) were made up to 4 ml by addition of 0.1 M tris buffer, pH 8.2 containing 0.02 M CaCl₂ and, with BAPNA (0.435 mg/ml water), were preincubated separately for 15 minutes at -4°C, 6°C, 25°C or 37°C. Then 4 ml of the BAPNA solution was added to the trypsin solution and the mixture incubated for 10 minutes. Hydrolysis was stopped with 1 ml of 30% acetic acid. Blanks were prepared in the same way, but without trypsin. The optical density of the samples was measured in a Zeiss spectrophotometer at 410 nm. The blank values were subtracted from the readings.

For the inactive renin studies, plasma samples were preincubated for 1 hour at the four temperatures and a range of trypsin concentrations were added. The trypsin reaction was stopped after 1 minute by addition of maleic acid and PMSF (table 1).

Trypsin and Urinary Kallikrein Hydrolysis of Acid-Treated Plasma

We dialyzed 50 ml of EDTA plasma in 3-ml aliquots at 4°C for 24 hours against 50 mM glycine-HCl, pH 3.3, containing 100 mM NaCl and 3 mM EDTA. The plasma was then adjusted to pH 7.4 at 4°C with 5 ml 4M tris HCl and approximately 0.5 ml 2N NaOH and 1-ml aliquots were preincubated for 1 hour at 25°C. Trypsin was then added to obtain the concentrations illustrated in figure 5 and the incubations were carried out for 1 hour at 25°C. Control plasmas without trypsin were also included in the study.

In another study highly purified urinary kallikrein, 360 µg/ml (specific activity 1094 µg equivalents bradykinin/min/mg at pH 8.5 and 37°C), was added in volumes ranging from 2.5 to 25 µl to 0.5 ml of acid-treated plasma. The urinary kallikrein was purified 833-fold and antibodies to it gave a single precipitin band on Ouchterlony double immunodiffusion when tested against pure antigen or against crude urinary proteins.

Incubation for renin assay of acid-treated plasma was carried out as described in table 1, except that only 60 µl of maleic acid was required to adjust the plasma from pH 7.4 to 5.7. In addition, 80 µl of par-
plasminogen was added prior to renin incubation to replace that which was destroyed during pH 3.3 dialysis.

**pH Optima of Renal Renin, Inactive Renin (Cryo) and Inactive Renin (Trypsin)**

Renal renin, prepared according to Haas et al., had a final concentration of 1.6 GU/ml. Of this solution 20 µl was added to 1 ml of plasma containing 4 mM diisopropyl phosphofloridate which had been adjusted to a wide range of pH values (17 points between 4.5 and 8.5). These were incubated for 10 minutes at 37°C, without shaking. The reaction was stopped by chilling. The samples were diluted 25-fold with chilled tris buffer, pH 7.4, prior to radioimmunoassay.

For determination of the pH optimum of inactive renin (cryo) and inactive renin (trypsin), a pool of EDTA plasma with endogenous PRA of less than 1 ng/ml/hr was cryoactivated or trypsin-activated. The renin incubation was then carried out in duplicate for 1 hour at 37°C (no shaking) in the presence of PMSF at the pH levels illustrated in figure 4.

**Urinary Kallikrein Measurements**

Urinary kallikrein was measured in 50 µl of dialyzed urine taken from a complete 24-hour collection. The method is highly specific and measures the rate of hydrolysis of Pro-Phe-Arg-(3H) benzylamide at pH 9.5. Under these conditions, 20 times more trypsin and 700 times more plasmin than urine kallikrein is required to hydrolyze 10% of the substrate. Alpha-chymotrypsin, urokinase and thrombin were unable to hydrolyze the substrate at concentrations 1000-fold higher than urine kallikrein. Ryan and co-workers have shown that there is only one protein in urine capable of hydrolyzing Pro-Phe-Arg-benzylamide at pH 9.5 and that protein is indistinguishable from human urinary kallikrein in terms of its chromatographic behavior and its reactivity with antibodies to pure kallikrein. In addition, we have found a direct correlation between the amidase assay and a direct radioimmunoassay for urinary kallikrein. For normal subjects, urinary kallikrein averaged 0.90 ± 0.15 (SEM) nmol/day by radioimmunoassay and 704 ± 83 units/day by amidase assay. For hypertensive patients, these values
Figure 3. Effect of trypsin concentration and incubation temperature on rate of hydrolysis of benzoyl-d, l-arginine-p-nitroanalide HCl (BAPNA) (left) and on the conversion of prorenin to active renin in plasma (right). For BAPNA hydrolysis, the rate of release of p-nitroanaline was 17 times faster at 37°C, 10 times faster at 25°C and 2.4 times faster at 6°C than at -4°C. In contrast, activation of inactive renin was fastest at -4°C and was only 60%, 80% and 90% of this rate at 37°C, 25°C and 6°C, respectively. A 15-minute incubation was used for BAPNA and a 1-minute period for inactive renin hydrolysis. OD = optical density.

Figure 4. The pH optimum of cryoactivated or trypsin-activated inactive renin was virtually identical to that of renal renin. The pH optimum for renal renin is the solid line on each panel. The open circles represent data for cryoactivated plasma renin and the closed circles are data for trypsin-activated plasma renin. For the renal renin studies 0.032 GU/ml were added to a pool of human plasma with low renin activity which was used as the source renin substrate. Incubation was carried out for 10 minutes at 37°C. For cryoactivated and trypsin-activated inactive renin, a pool of plasma was used with a plasma renin activity of less than 1 ng/ml/hr at pH 5.7 and 37°C. After activation the total renin was 9.5 ng/ml/hr for cryoactivation and 19 ng/ml/hr for trypsin activation.
averaged 1.36 ± 0.23 nmol/day and 1275 ± 155 units/day, respectively. The Spearman rank correlation coefficient between the two assays was 0.72 for 38 normal subjects and 0.75 for 30 hypertensive patients (unpublished observations).

One unit of urinary kallikrein is the amount of enzyme required to hydrolyze Pro-Phe-Arg-(3H) benzylamide at an initial rate of 1%/min at 37°C.

Subjects Participating in the Study

The comparison of trypsin and cryoactivation was carried out in 60 normal subjects (26 females, 34 males; 35 whites, 25 blacks) who participated in a longitudinal follow-up study of the renin-aldosterone system at the Hypertension Center of The New York Hospital-Cornell Medical College. Urinary kallikrein was measured in five of the normal subjects and in 17 untreated patients with essential hypertension who attended the Hypertension Center. Informed consent was given.

All subjects collected a complete 24-hour urine sample and at the end of the collection period 20 ml of blood was collected before noon in the seated position, after a 2-hour period of ambulation.

Hageman factor and Fletcher factor deficient plasmas were purchased from George King Biochemical Inc., Overland Park, Kansas.

Results

Effect of Trypsin Concentration, and Incubation

Temperature and Time on Activation of Inactive Renin

In figure 2, renin measurements are presented for a pool of plasma following incubation with five trypsin concentrations, ranging up to 1000 μg/ml, for five time periods up to 14 hours and at -4°C, 6°C, 25°C and 37°C. These studies were also carried out for 24, 30 and 48 hours, but the data are not shown.

Control PRA averaged 2.3 ng/ml/hr. At -4°C, in the absence of trypsin there was a slow increase in renin activity of 0.13 ng/ml/hr each hour beginning after 6 hours of incubation. There was no increase in renin activity at any of the other temperatures. At trypsin concentrations above 600 μg/ml and at all temperatures tested except 37°C, a plateau of renin activity was reached between 1 and 14 hours. At lower concentrations of trypsin, renin continued to be formed throughout the entire 48 hours. At all temperatures and at all concentrations of trypsin tested, the renin increase in the first minute was at least as great as the additional increase during the following 3 hours.

There was no destruction of renin in EDTA plasma at temperatures below 26°C and at trypsin concentrations 1 mg/ml or below. In other studies we have observed some destruction of human plasma renin by trypsin at -4°C at concentrations above 2 mg/ml (unpublished observations).

In contrast, maximum renin activity was never achieved at 37°C. At 37°C there was always an initial increase in renin for up to 3 hours. After 6 hours, renin activity fell and by 48 hours it had returned almost to baseline. However, destruction of renin at 37°C was not primarily caused by trypsin. In the control sample without trypsin, renin activity also fell (fig. 2) and, in another study, a pool of plasma with high endogenous renin activity showed a gradual decline in renin activity from 20 to 5 ng/ml/hr over 14 hours* in the absence of trypsin. This fall was not inhibited by DFP or PMSF. All of the trypsin studies were carried out in pH unadjusted plasma collected in EDTA. After freezing, these plasmas have pH values close to 8.0. Destruction of renin at 37°C was probably due to instability of renin at 37°C at pH values above 8.0. Since the pH was the same at the other temperatures tested (-4°C, 6°C, 25°C), renin appears to be quite stable in plasma at pH 8 as long as the temperature does not increase above 25°C.

At all concentrations of trypsin that did not cause maximum activation of inactive renin, the rate of renin activation increased as the temperature was lowered (fig 3, right). This unusual effect of temperature was in striking contrast to the effect of low temperatures on hydrolysis of BAPNA by trypsin. As expected, release of p-nitroaniline by trypsin was fastest at the highest temperatures (fig. 3). At 37°C, 25°C and 6°C the rates of product formation were respectively 17, 10 and 2.4 times the rate at -4°C. In contrast, at 37°C, 25°C and 6°C, the rate of renin generation from inactive renin was 0.6, 0.8 and 0.9 times the rate at -4°C.

Characteristics of Renin Released by Trypsin

Studies were carried out to evaluate whether trypsin activation, like acid activation and cryoactivation, increased the renin content of plasma. For a kinetic analysis, eight concentrations of partially purified human renin substrate were added to 2.5-fold diluted plasma. Control plasma had an apparent Michaelis constant (Km) of 1.2 μg Ang I/ml and maximum velocity (Vmax) of 12.7 ng/ml/hr, while the same plasma after trypsin activation had a Km of 1.1 μg Ang I/ml and maximum Vmax of 65.9 ng/ml/hr. Thus, trypsin increased the renin content without any apparent change in either the affinity for homologous renin substrate or in the concentration of competitive activators or inhibitors of renin.

Trypsin has been used to hydrolyze tetradecapeptide (TDP) from horse renin substrate but has never been shown to affect human renin substrate. Since TDP can be hydrolyzed to angiotensin I by plasma pseudorenin, a highly potent enzyme with a pH optimum of close to 4.5, it was possible that the observed increase in the rate of angiotensin formation after trypsin treatment was not due to renin but to the reaction of pseudorenin with TDP. We have shown that this is unlikely since there was no increase in the blank following trypsin activation. Thus, in 12 activated plasmas with a mean total renin of 8.0 ± 0.5 ng/ml/hr the blank was 1.2 ± 0.1 ng/ml compared to 2.0 ± 0.3 in seven plasmas with a mean active renin of 8.4 ± 0.7 ng/ml/hr. Tetradecapeptide, but not renin sub-
strate, binds to angiotensin I antibodies and the blank would have increased if TDP had been formed. In addition, we determined the pH optimum of trypsin-activated renin and compared it to that of renal renin and cryoactivated renin. For all three the pH optimum was consistently between 5.5 and 6.0 and the shape of the curves were quite similar (fig. 4). There was no downward shift of the pH optimum. We also found that dialysis of trypsin-activated plasma did not affect either the renin substrate concentration or the rate of angiotensin generation, whereas dialysis effectively removed TDP added to plasma. When TDP was added to plasma the renin substrate measurement increased from 1333 ng/ml to 2598 ng/ml and fell to 1338 ng/ml after 24 hours dialysis against 5 liters of buffer. Thus, because the pH optimum did not fall and there was no change in renin substrate concentration following dialysis, and because the blank did not increase disproportionately after trypsin activation, there is no evidence that 1 mg/ml trypsin hydrolyzes tetradecapeptide from human renin substrate in plasma at -4°C.

Effect of Trypsin on Acid-Treated Plasma

We have previously shown that almost two-thirds of the inactive renin in plasma remains inactive after pH 3.3 dialysis. If acidified plasma is adjusted to pH 7.4, almost no activation by endogenous proteases occurs during the first 6 hours at 25°C. Therefore, during this latent period, the potency of proteolytic enzymes for activating inactive renin can be tested.

In Figure 5 the potency of trypsin in converting inactive renin at 25°C is compared in whole plasma and acid-treated plasma. Maximum activation was achieved by 200 μg/ml trypsin in acid-treated plasma whereas 800 μg/ml was required in whole plasma. However, at 1000 μg/ml trypsin destroyed renin in acid-treated plasma at 25°C. More than 2000 μg/ml trypsin is required to begin to destroy renin in whole plasma at 25°C (unpublished observations). There was no difference in the rate of activation of renin at -4°C or 25°C in acid-treated plasma at concentrations of trypsin below 100 μg/ml, but above this concentration renin was destroyed by trypsin at -4°C.

Effect of Urinary Kallikrein on Acidified Plasma

Urinary kallikrein, like trypsin, activated inactive renin in acid-treated plasma (fig. 6). There was a concentration-dependent increase up to approximately 12 μg/ml (fig. 6, upper panel). As illustrated in the lower panel, prolonging the incubation time was not as effective as doubling the concentration of urinary kallikrein. There was little further increase in renin activity between 1 and 2 hours. This suggests that factors that inactivate urinary kallikrein may survive acid treatment.

Lack of Effect of Pancreatic Kallikrein in Acidified Plasma

Since it has been reported that hog pancreatic kallikrein activates inactive renin in human amniotic fluid but not in human plasma, we tested the effect of

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**Figure 5.** Comparison of concentrations of trypsin required for activation of inactive renin in EDTA plasma or in plasma which was dialyzed to pH 3.3 for 24 hours at 4°C and then adjusted to pH 7.4 at 4°C. Samples were preincubated at either 25°C or -4°C for 1 hour before addition of trypsin. Much less trypsin was required to activate renin in acid-treated plasma than in untreated plasma, but in acid-treated plasma destruction of renin occurred at much lower concentrations of trypsin.
Figure 6. Effect of urinary kallikrein concentration, incubation temperature and incubation time on activation of inactive renin in acid-treated plasma. During a 1-hour incubation at 25°C, maximum activation occurred at concentrations between 10 and 18 ng/ml (upper panel). Prolonging the incubation period beyond 1 hour caused almost no further activation (lower panel).

Comparison of Trypsin Activation, Cryoactivation and Urinary Kallikrein Activation of Inactive Renin

There was a highly significant direct relationship between inactive plasma renin in 60 normal subjects measured by trypsin or cryoactivation, $r = 0.65$, $p < 0.001$. However, cryoactivated inactive renin averaged only $3.8 \pm 2.1$ (SD) ng/ml/hr, while the trypsin activated measurement was $13.4 \pm 1.9$ ng/ml/hr. In these normal subjects active renin averaged $2.3 \pm 1.7$ ng/ml/hr. Thus inactive renin (trypsin) was more than fivefold higher than the active plasma renin. There were three white subjects (two men, one woman) whose inactive plasma renin did not cryoactivate, but did trypsin activate.

Urine kallikrein activation of acid-treated plasma, and trypsin activation of whole plasma, gave close to identical results (table 2) in pools of plasma from hypertensive patients and in plasma from patients with either Hageman factor or Fletcher factor (pre-kallikrein) deficiency.

Relationship Between Urinary Kallikrein Excretion and the Proportion of Active Renin in Plasma

These studies were carried out in 22 subjects who had renin values less than 5 ng/ml/hr on a random sodium intake. There was a direct relationship ($r = 0.46$, $p < 0.05$) between the daily rate of urinary kallikrein excretion and the active renin level expressed as a percentage of the total renin measurement after cryoactivation (fig. 7). When urinary kallikrein excretion was 1200 units/day the proportion of active renin in plasma was close to 19%, and this fell to about 7% when urinary kallikrein excretion was 200 units/day.

The proportion of active renin in plasma was calculated for 35 white and 25 black normal subjects. Whites had a higher proportion of active plasma renin ($17.7\% \pm 2.4$ SE) than blacks ($10.2 \pm 1.4, p < 0.02$), but there was no significant difference in active renin ($2.6 \pm 0.3$ vs $2.0 \pm 0.3$ ng/ml/hr) or inactive renin measurements ($15.5 \pm 2.1$ vs $16.6 \pm 2.1$ ng/ml/hr).

Discussion

In the present study we have defined conditions for reproducible activation of inactive plasma renin by trypsin. We have shown that the incubation temperature must be maintained below $37^\circ$C to avoid destruction of renin in unadjusted plasma collected in EDTA. We have also shown that activation of inactive plasma renin by trypsin is more rapid at $-4^\circ$ than at $6^\circ$, $25^\circ$ or $37^\circ$C. This is because certain endogenous inhibitors of trypsin are less effective at reduced temperatures, so that less trypsin is required to overcome them in cold plasma. Therefore, we routinely activate inactive plasma renin with 1 mg/ml trypsin during a 1-hour incubation at $-4^\circ$C.

When plasma is dialyzed to pH 3.3, many endogenous plasma inhibitors are destroyed and, in acid-treated plasma, only $100 \mu$g/ml trypsin was required for complete activation at $-4^\circ$C (fig. 5). However, some inhibitors survive acid treatment (unpublished data), and therefore trypsin activation of inactive renin in acid-treated plasma was no more effective at $25^\circ$C than at $-4^\circ$C (fig. 5). Very high concentrations of trypsin (more than 2 mg/ml) destroy renin in whole plasma (unpublished observations) but destruction occurred in acid-treated plasma at trypsin concentrations of 1 mg/ml at $25^\circ$C (fig. 5) and 200 $\mu$g/ml at $-4^\circ$C.
Theoretically, trypsin could activate renin indirectly via its ability to activate one of the coagulation or fibrinolytic enzymes. In this study we demonstrated that neither Hageman factor nor prekallikrein (Fletcher factor) participate in the activation by trypsin since activation occurred in plasmas deficient in these factors (table 2).

We have confirmed that trypsin does not release TDP from renin substrate in human plasma. However, we have found (Gallagher et al., unpublished observations) that trypsin treatment of dog plasma can produce a dialyzable form of dog renin substrate and at the same time it causes a shift in the pH optimum of renin to 4.5. These findings suggest that in dog plasma, trypsin can form TDP which is then hydrolyzed by a pseudorenin-like enzyme. In the present study, we showed that the pH optimum of trypsin-treated human plasma is the same as that of renal renin and cryoactivated renin (fig. 4). In addition, we showed that, while dialysis can remove TDP

### Table 2. Comparison of Total Renin Measured by Urinary Kallikrein or Trypsin

<table>
<thead>
<tr>
<th>Plasma source</th>
<th>Total renin (ng/ml/hr)</th>
<th>UK/Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UK* (0 µg/ml)</td>
<td>UK* (6 µg/ml)</td>
</tr>
<tr>
<td>Pool #1</td>
<td>5.1</td>
<td>7.1</td>
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<td>Pool #1</td>
<td>4.3</td>
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<tr>
<td>Pool #1</td>
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<td>XII def†</td>
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<td>#1</td>
<td>4.8</td>
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</tr>
<tr>
<td>#2</td>
<td>4.1</td>
<td>10.1</td>
</tr>
<tr>
<td>Fletcher def†</td>
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<td></td>
</tr>
<tr>
<td>#1</td>
<td>8.5</td>
<td>28.3</td>
</tr>
<tr>
<td>#2</td>
<td>8.8</td>
<td>29.5</td>
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*Urinary kallikrein (UK) was added to plasma that had been previously dialyzed to pH 3.3 then adjusted to pH 7.4.
†Trypsin was added to untreated plasma collected in EDTA.
‡Factor XII deficient (def) plasma was from two different patients. Fletcher factor deficient plasma was from two different bleedings of the same patient.
added to plasma, dialysis does not affect either the endogenous renin substrate concentration or the increase in renin activity that occurs in human plasma after incubation with trypsin.

Cryoactivation of inactive renin resulted in only about one-third of the increase in plasma renin caused by trypsin. However there was a highly significant relationship between cryoactivated and trypsin-activated inactive renin in normal subjects and in pregnant women, suggesting that both procedures activate the same pool of inactive renin. To date, trypsin activation appears to be the method of choice for reproducibly activating inactive renin, but caution must be used to avoid laboratory contamination, since the concentrations required to overcome plasma inhibitors are extraordinarily high. Also, different concentrations of trypsin may be required in patients who have abnormal trypsin inhibitor concentrations.

Urinary kallikrein activated inactive renin in acid-treated plasma to the same maximum as trypsin activation of inactive renin in whole plasma (table 2). Unlike trypsin, urinary kallikrein was more potent at 25°C than at −4°C in acid-treated plasma (fig. 6). Maximum activation could be achieved at 25°C by between 6 and 12 μg/ml plasma, concentrations which have virtually no effect in whole plasma. Increasing the incubation time with submaximal concentrations of kallikrein beyond 1 hour at 25°C did not result in much further activation of inactive renin, which suggests that the enzyme may be inhibited or destroyed by acid-treated plasma. Thus it is possible that much lower concentrations of urinary kallikrein would activate purified inactive renin. As with trypsin, neither Hageman factor nor plasma prekallikrein were required for urinary kallikrein activation.

In this series of studies we observed a direct relationship between urinary kallikrein excretion and the proportion of active renin in plasma (active/total). In addition, normotensive blacks had a lower proportion of active plasma renin than whites, and they are reported to have significantly lower excretion rates of urinary kallikrein. These observations could be the result of a biochemical relationship between renal kallikrein and the in vivo activation of inactive renin. Histological and stop-flow studies have suggested that renal kallikrein is located in the cells lining the distal convoluted tubule extending from the juxtaglomerular apparatus, where renin is synthesized, to the collecting duct. These reports have not directly examined the possibility that kallikrein might be present in the renin-secreting cells of the juxtaglomerular apparatus. Such a possibility is supported by the report that renin and kallikrein are in the same microsomal fraction of rat kidney extracts and also by the observation that a patient with a renin-secreting tumor had very high urinary kallikrein excretion.

Although much more work is required to determine whether renal kallikrein converts inactive to active renin in vivo, its intrarenal location could interfere with the isolation of large molecular weight forms of renin from the kidney. Thus, Slater and Haber found that the protease inhibitor PMSF, in combination with EDTA, dimercaprol and 8-OH quinoline, was required to preserve the large molecular weight form of renin in extracts of human kidney. In addition, while the prorenin-like substance in plasma is completely inactive (unpublished data), the large molecular weight forms of renin are at least 10% as active as the 40,000 molecular weight form, raising the possibility that partial activation may have occurred during extraction.

In summary, the present study defines conditions for reproducible activation of inactive plasma renin by trypsin. Like cryoactivation, trypsin is most effective at low temperatures, thus supporting the concept that cold renders ineffective one or more inhibitors of endogenous neutral serine proteases. In addition, we have shown that urinary kallikrein activates inactive plasma renin to the same degree as trypsin, both in normal plasma and in plasma deficient in Hageman factor or prekallikrein. Finally, the observation that the proportion of inactive renin in plasma is directly related to the urinary excretion of kallikrein lends support to the concept of an intrarenal link between kallikrein and renin.

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