Direct Radioimmunoassay of Human Renin
Comparison with Renin Activity in Plasma and Amniotic Fluid

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SUMMARY Human plasma and amniotic liquid were activated by dialysis at pH 3.3. Then, renin before and after acidification was determined by two methods: enzymatic activity measurement, and direct radioimmunoassay. The identity between nonactivated and activated renin in plasma and amniotic fluid on the one hand, and pure renin on the other, was demonstrated by the dilution curves in radioimmunoassay. After acidification, mean plasma renin activity in 17 patients with high renin activity rose from 26.8 ± 11.7 pmoles A I ml⁻¹ h⁻¹ to 67.9 ± 29.3 pmoles A I ml⁻¹ h⁻¹, whereas the mean renin concentration tested by direct radioimmunoassay remained constant at 13.8 ± 10.5 and 14.8 ± 11.2 fmol/ml before and after acidification respectively. In amniotic fluid, renin activity increased from 9.7 to 227 pmoles angiotensin I/ml/h, but the renin concentration did not change. Direct radioimmunoassay of renin may therefore be considered as measuring total renin, regardless of its enzymatic activity. In 12 hypertensive patients undergoing bilateral renal-vein catheterization, the direct measurement of renin was very significantly correlated to the non-activated (r = 0.883) and activated renin values (r = 0.963). (Hypertension 2: 465-470, 1980)

KEY WORDS human renin radioimmunoassay · renin activation · amniotic fluid · binephrectomized patient renin · pepstatin

FIFTEEN years after the initial description of the methods currently used for measuring renin (E.C.-3-4-99-19) in human plasma and despite their successive improvements, the investigation of this enzyme is still limited by many methodological problems. Thus, plasma renin activity (PRA) is not measured in the presence of a constant excess of renin substrate. To control this variable element, heterologous substrates extracted from sheep, or oxen are commonly used, but compared to human angiotensinogen, they introduce differences in enzymes kinetics. The amount of angiotensin I released from renin substrate through the enzymatic property of renin in plasma or amniotic fluid can be greatly changed by several processes generally designated as “renin activation,” i.e., refrigeration, acidification, or addition of proteolytic enzymes. Such activation is not necessarily linked to conversion of the forms of higher molecular weight renin in human plasma and kidney into lower molecular weight forms, but could be due to a minor structural change in the renin molecule, or to the destruction of a renin inhibitor.

The preparation of highly specific human renin antibodies offers new ways of avoiding these problems. With these antibodies, we prepared an affinity chromatography gel in order to separate human angiotensinogen from human renin in a single step. We also used antibodies to characterize renin in plasma from binephrectomized patients. Finally, we set up a direct renin radioimmunoassay and compared the number of immunoreactive molecules present in plasma and amniotic fluid to the enzymatic activity before and after activation.

Methods
Preparation of Renin Substrate
Renin-free human angiotensinogen was prepared by affinity chromatography using immobilized anti-human-renin-antisera. This immunoadsorbant was obtained by coupling 10 ml of rabbit anti-human-renin-antiseraum brought to pH 8.0 with 100 ml Sepharose 4 B (Pharmacia) according to March et al. After overnight shaking at 4°C, the gel was washed with 1 M glycine, water, 2 M acetic acid, 6 M urea, and 0.1 M phosphate buffer at pH 7.5. The efficiency of the gel was checked by its ability to bind

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95% radioactive iodinated human renin. Human plasma was obtained from women taking oral contraceptives; 130 ml of plasma were added to 130 ml of 0.2 M phosphate buffer, pH 7.5. The sample was then gently shaken for 3 hours at 4°C together with 90 ml Sepharose-anti-human-renin-antiserum. After 5 minutes of centrifugation, the supernatant was dialyzed overnight against 0.1 M phosphate citrate buffer pH 5.7 containing 2% disodium ethylenediamine tetraacetate acid. The 230 ml sample was then stored at −24°C in 5 ml aliquots.

Renin Activation Procedures

Activation of renin in amniotic fluid or plasma was performed by 24 hours of dialysis at 4°C against 0.1 M citrate-phosphate buffer, pH 3.3, followed by 24 hours of dialysis against 0.1 M phosphate buffer, pH 7.4. Control samples were dialyzed for 48 hours at pH 7.4. Since acidification destroys renin substrate, the angiotensinogen concentration in acidified plasmas was restored by adding 275 pmoles of human angiotensinogen (see above). The same amount of angiotensinogen was used for renin enzyme activity determination in amniotic fluid.

Radioimmunoassay (RIA)

Plasma Renin Activity

PRA was determined by the amount of angiotensin I generated at the optimum pH, pH 5.7 and measured by RIA with a standard curve ranging from 5 to 80 pg; 250 μl plasma or 100 μl amniotic fluid were incubated for 30 minutes at 37°C in a total volume of 500 μl. Results are expressed in pmole angiotensin I generated per milliliter (ml) per hour.

Angiotensinogen Determination

An exhaustion method that used cadaver kidneys as a source of renin was used to measure angiotensinogen concentrations, which were expressed in angiotensin I equivalent generated per milliliter, on the assumption that 1 mole of angiotensinogen generates 1 mole of angiotensin I.

Direct Renin Radioimmunoassay

Direct renin RIA was performed as described on 100 μl plasma or 10 μl amniotic fluid. Purity of the iodinated renin was checked by Agarose-Acrylamide gel filtration (fig. 1), and by isoelectric focusing on polyacrylamide gel electrophoresis according to Doerr and Chrambach. A single symmetrical peak was obtained with an isoelectric point 6.6. Results were expressed in fmole of enzyme. Variance analysis was performed to demonstrate the linearity of the standard curve and of the displacement curves for activated and nonactivated renin, and the slopes of these curves were compared.

Inhibition of Plasma Renin by Human Renin Antibodies

Plasma (250 μl) from binephrectomized patients were preincubated for 24 hours at 4°C with an excess of antirenin antiserum (25 μl of a 1/100 dilution) at pH 5.7. Then PRA was determined at pH 5.7 in a 500 μl volume, as described above. In control experiments, preincubation was run in the absence of antiserum. It was verified that under these conditions the antiserum concentration used completely inhibited standard human renal renin (Medical Research Council) and renin from normal plasma.
Results

Preparation of Renin-Free Substrate

Angiotensinogen prepared by affinity chromatography was obtained with 52% recovery whereas renin was completely eliminated (table 1). No angiotensin I formation could be detected even when incubation lasted for as long as 6 hours. To demonstrate that activated and nonactivated renin were removed from plasma by this affinity chromatography, plasma eluted from the gel was acidified and then incubated with exogenous angiotensinogen. The absence of angiotensin I generation demonstrates that exposure to immobilized antirenin antibodies removes both nonactivated and activated renin from plasma. It also shows that acidification of such plasma is not able to unmask an angiotensin I-forming enzyme distinct from renin.

Direct RIA of Activated and Nonactivated Renin

Figure 2 illustrates the dilution curves, in direct RIA, for plasma and amniotic liquid before and after acidification. The slopes of these curves are identical to the standard curve constructed with pure renin. This confirms the immunoreactive identity of renin (activated or nonactivated) in plasma and amniotic liquid with pure enzyme. Renin inhibitor pepstatin did not affect the RIA of renin at a 10^{-5} M concentration. Thus, the standard curve constructed in the presence or absence of this pepstatin concentration was indistinguishable.

The influence of acid activation of plasma renin on the generation of angiotensin I is shown in figure 3. There was a 253% mean increase in renin enzymatic activity after acidification of the plasma from 17 high-renin hypertensive patients. By contrast, the mean level of the immunoreactive renin tested by direct RIA did not rise after acidification. The same observation applied to plasma obtained before surgery from a patient suffering from a renin-secreting tumor which allowed purification of human renin. 20 In this subject, 175.3 pmoles of angiotensin I ml^{-1} h^{-1} were generated before acidification and 2153.8 pmoles ml^{-1} h^{-1} afterward. There were 1605 fmoles ml^{-1} of renin found before and 1456 fmoles after acidification.

In amniotic fluid, a dramatic increase in renin enzymatic activity followed acidification (from 9.7 to 227 pmoles angiotensin I/ml/hr, but immunoreactive renin remained unchanged (457 and 462 fmoles ml^{-1} before and after).

Correlation Between Enzymatic Assay and Direct RIA

In figure 4, renin enzymatic assay is plotted against renin direct RIA in 36 plasmas from 12 patients undergoing bilateral renal-vein catheterization. Renin secretion was stimulated before catheterization by sodium depletion (four cases) or captopril treatment (eight cases). To avoid inadvertent cryoactivation,

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### Table 1. Renin-Free Angiotensinogen

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total angiotensinogen (pmole)</th>
<th>Yield (%)</th>
<th>Total renin (pmole Al/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>130</td>
<td>440</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>After affinity chromatography</td>
<td>200</td>
<td>226</td>
<td>52</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

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**Figure 2.** Direct radioimmunoassay of human renin in plasma and amniotic fluid before and after acidification. The abscissa gives the logarithm scale for standard human renin (fmol) or sample volumes (μl); the ordinate, [B/Bo] x 100, is the percentage of bound radioactivity in the presence and absence of unlabeled renin. Pure renin (1.12 to 9 fmol is denoted by connected black circles; amniotic fluid before acidification by connected black squares, and after acidification (2.5 to 15 μl) by connected open squares. Plasma before acidification is given by black triangles and afterward by open triangles (acidification = 1.25 to 7.5 μl). This high renin plasma was obtained from a patient suffering from a renin tumor before surgery (see text).
Figure 3. Mean variation in plasma renin before and after acidification. Renin was measured by two methods: enzymatic assay (pmol angiotensin I generated/ml/hr) and direct radioimmunoassay (fmol/ml) in plasma from 17 high renin hypertensive patients before (NA) and after (A) acidification.

Figure 4. Correlations between the enzymatic assays and the radioimmunoassay of plasma renin. Plasma renin measured by radioimmunoassay was plotted against Angiotensin I liberated by nonactivated and activated plasmas.

Each plasma sample was immediately divided in several aliquots for the measurements of activated, and nonactivated renin, and for direct renin RIA. Both activated and nonactivated renin were very significantly correlated to immunoreactive renin ($r = 0.883$, $n = 36$; after activation: $r = 0.963$, $n = 35$). Activated and nonactivated renin were also significantly correlated ($r = 0.538$).

Inhibition of PRA in Anephric Patients

The lack of sensitivity of the present direct RIA did not allow measurements of renin in plasma from binephrectomized patients. The PRA of such three plasmas was 0.953, 0.123 and 0.253 pmoles angiotensin I/ml/hr. The similarity between renin from normal and binephrectomized patient plasma was demonstrated by complete inhibition of PRA in such plasmas preincubated with an excess of antirenin antiserum.

Discussion

The angiotensinogen preparation described here enables selective removal of renin from plasma in a rapid reproducible way. The same affinity gel was used another three times to prepare other batches of renin-free substrate with similar recoveries. Since PRA measurement methods usually underestimate the renin concentration in plasma, it is worth working out a method that provides renin-free angiotensinogen and is suitable for the measurement of renin in the presence of a constant excess of homologous substrate. Use of an aminohexyl-pepstatin-agarose column or a concanavalin A-Sepharose column was suggested earlier for this purpose; nevertheless, although the pepstatin column was very effective in removing a large amount of renin, affinity chromatography using antirenin antibodies made possible more complete renin extraction, when a small amount of endogenous enzyme was present in plasma. This renin-free angiotensinogen should be used rather than plasma from binephrectomized subjects, since such plasma frequently contains a small amount of renin-like enzyme, even in the absence of activation.

In our study, renin activity found in plasma from binephrectomized patients was completely inhibited by specific renin antibodies. It brings another argument in favor of the presence of circulating renin similar to renal renin in such patients.

For the first time, direct renin RIA allows quantification of the number of immunoreactive molecules in human plasma. The present results emphasize the very low renin concentration in plasma (fmole range/ml). The assay used here is not yet sufficiently sensitive to detect renin in normal patients, however, and our studies are currently limited to patients with high PRA, i.e., more than 20 pmoles angiotensin I ml$^{-1}$ hr$^{-1}$ after acidification. The renin activation by acidification previously reported both in plasma and amniotic liquid is confirmed here. This activation process varies from one plasma to another, but the
mean percentage increase of renin activity observed in our high renin subjects is of the same order of magnitude as the increases observed by other authors, using several activation methods.15, 43

In the absence of activation, PRA measurement is generally correlated to circulating angiotensin II.38 This correlation suggests that the PRA level is a valuable and practical index to the state of activation of the renin-angiotensin system. This index is the main element supporting our present concepts of the influence of posture, sodium diet, and drugs like beta-blockers on the degree of response of the renin-angiotensin system. Nevertheless, measurement of the number of immunoreactive renin molecules secreted by normal or pathological kidneys under different conditions remains necessary for complete investigation of this system. Until the present RIA was worked out, however, such measurement was impossible.

In this study of 36 venous plasma samples from renal veins and inferior vena cava, we found a very significant correlation between enzymatic and immunoreactive renin, in activated plasmas and nonactivated plasmas. In mouse plasma as in human plasma, Michelakis et al.45 and Malling and Poulsen46 found a high correlation between nonactivated renin and immunoreactive renin.

The potential usefulness of direct renin RIA as a tool for determining true renin secretion by the kidney is demonstrated by the renin level found in the plasma of our patient with a renin-secreting tumor. In such cases, when the amount of renin really present in the circulation is very high, the inappropriate conditions for the enzymatic reaction lead to underestimation of renin in the enzymatic assay of activated and nonactivated plasmas. High renin is not a rare condition in patients with severe hypertension, and renin-secreting tumors among such patients would certainly be more easily detected by the renin RIA. Two hypotheses can be suggested to explain the fact that acidification increases renin enzymatic activity but leaves its immunoreactivity unchanged:

1. Kinetic changes in the renin-substrate reaction (by the removal of an inhibitor or the unmasking of an activator).
2. Changes in the renin molecule that passes from an inactive to an active state without any change in the total number of immunoreactive centers.

According to the first hypothesis, there would be no variation in renin structure during activation but an increase in the affinity of renin for its substrate. Such a change should lead to a variation in kinetics during the enzymatic assay of renin. The direct renin RIA, on the other hand, would remain constant, whether or not an inhibitor was present, as demonstrated by the absence of any change in the renin RIA standard curve in the presence of the pepstatin concentration (10^-4 M) which inhibits the enzymatic activity of human renin.41 The hypothesis of a kinetic change caused by inhibitor removal has been investigated in plasma by Derkx et al.48 and Atlas et al.49 and in amniotic fluid by Shulkes et al.50 Morris,19 and Ito et al.51 None of these authors found any significant modification in Km after renin activation, which rules out the hypothesis of a competitive inhibitor removal. However, these results fit with the hypothesis of a noncompetitive inhibitor removal.

Also attractive is the hypothesis of renin activation due to a change in renin conformation, which would require the antibody to make no distinction between the two renin forms. In our present study, the fact that the dilution curves are identical in renin RIA before and after acidification argues in favor of this second hypothesis. Our data are consistent with a possible change in conformation that does not alter immunoreactivity. Such an assumption is in good agreement with the results of Boyd,12 who only found a minor change in molecular weight between nonactive and active renin in plasma. Furthermore Shulkes et al.52 Morris,19 and Ito et al.51 reported close similarity of the physicochemical properties of nonactivated and activated renin in amniotic fluid. The discrepancy between immunoreactivity and biological activity is rather a common feature of polypeptides and was the origin of the discovery of hormonal precursors.48 In the renin-angiotensin system proper, a discrepancy between immunological and enzymatic methods was pointed out by Egenna et al. They worked out an angiotensinogen RIA that measures both active angiotensinogen and inactive Des-angiotensin I-angiotensinogen and that under certain circumstances differs from the enzymatic assay.48

Another important application of renin antibodies is the characterization of angiotensin-forming enzymes found in biological fluids or extrarenal tissues. Our study clearly shows the close similarity and the immunological identity between amniotic and plasma renin. Even when the direct RIA cannot be applied due to the low amount of angiotensinogen I-angiotensinogen and that under certain circumstances differs from the enzymatic assay.48

In conclusion, the new methodology developed through the use of specific renin antibodies has helped to throw light on the renin-angiotensin system in plasma and tissues. More sensitivity in our present methodology is nevertheless needed to enable investigation of conditions under which renin is secreted in normal or low amounts.

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