BBA 66741

THE ACTIVATION OF RENIN IN HUMAN AMNIOTIC FLUID BY PROTEOLYTIC ENZYMES

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(Received June 6th, 1972)

SUMMARY

Renin (EC 3.4.4.15) in human amniotic fluid can be activated by the proteolytic enzymes pepsin (EC 3.4.4.1) and trypsin (EC 3.4.4.4). Furthermore, activation of renin at pH 3.3–3.6 is due to an acid-stable factor which is inactive at physiological pH, and has properties similar to the enzyme pepsin. The possible relationship between these findings and the secretion of renin by the kidney is discussed.

INTRODUCTION

Renin (EC 3.4.4.15) is a proteolytic enzyme, which is present in many tissues (Turrian[1]; Gould *et al.*[2]; Ferris and Mulrow[3]). High concentrations occur in kidney and human amniotic fluid (Brown *et al.*[4]). The concentration of renin can be measured by the rate of formation of angiotensin at pH 7.5 from sheep or human substrate (Skinner[5]). Exposure of human amniotic fluid or plasma to pH 3.3–3.6 greatly increases the concentration of renin measured at pH 7.5. This finding suggests that renin is irreversibly modified by low pH treatment (Lumbers[6]). Skeggs *et al.*[7] also observed that hog renal renin, prepared at pH 7.0, was converted to a second form of renin of higher specific activity by low pH treatment.

In the present study, the mechanism of activation of renin in human amniotic fluid at low pH was investigated. A preliminary account of part of this work has been presented by Morris and Lumbers[8].

MATERIALS AND METHODS

Amniotic fluid. Amniotic fluid was collected from patients following artificial rupture of foetal membranes, a procedure which induces the onset of parturition.

Buffers.

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pH 7.5 (0.18 M): 98.9 mM sodium phosphate, 5 mM EDTA, 76 mM NaCl.

pH 6.5 (0.16 M): 100 mM sodium phosphate, 1 mM EDTA, 58.6 mM NaCl.

pH 5.5 (0.16 M): 18.7 mM citric acid, 111 mM NaH_2PO_4, 1 mM EDTA, 29 mM NaCl.

pH 4.5 (0.16 M): 27.8 mM citric acid, 45 mM $Na_2HPO_4 \cdot 12H_2O$, 5 mM EDTA, 82.0 mM NaCl.

pH 3.3 (0.16 M): 50 mM glycine, 10 mM HCl, 5 mM EDTA, 95 mM NaCl.

pH 1.5 (0.16 M): 72.3 mM KCl, 32 mM HCl, 155.7 mM NaCl.

Bovine serum albumin buffer: pH-7.5 buffer containing 2% (w/v) bovine serum albumin (Commonwealth Serum Laboratories, Cohn Fraction V), 0.16 mM dimercaprol (Boots) and 3.4 mM 8-hydroxyquinolone.

[βAsp(NH_2)^1, Val^5]-Angiotensin II (Hypertensin, Ciba, Basle, Switzerland) stored at a concentration of 10 μg/ml in 0.15 M NaCl at —20 °C.

[Asp^1, Ile^5]-Angiotensin I (Schwarz BioResearch, Orangeburg, N.Y., U.S.A.) stored at a concentration of 2 μg/ml in bovine serum albumin buffer at —20 °C.

^{125}I-labelled Angiotensin I, iodinated by a modification of the method of Hunter and Greenwood[9], purified by the method of Haber *et al.*[10], and stored in bovine serum albumin buffer.

Antibody to angiotensin I. Immune goat serum was incubated at 56 °C for 30 min, EDTA (300 mM) added, and the serum diluted 1:200 with bovine serum albumin buffer.

Enzymes. Pepsin (EC 3.4.4.1) (Sigma, 3× crystallized), trypsin (EC 3.4.4.4) (Sigma, 2× crystallized), chymotrypsin (EC 3.4.4.5) (Sigma, 3× crystallized), and carboxypeptidase (Sigma, 2× crystallized in toluol), all stored in a desiccator at 5 °C.

Estimation of renin

In all experiments, amniotic fluid was dialysed in 8/32-inch Visking cellophane casings to a final pH of 7.5. The concentration of renin was determined at this pH by the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) at 37 °C from a constant amount of nephrectomized sheep plasma, which contains excess renin substrate but no renin (Skinner[5]). The addition of exogenous substrate is necessary since amniotic fluid contains little or no endogenous substrate (Skinner *et al.*[11]). Angiotensin was measured by bioassay or radioimmunoassay.

Bioassay. Following dialysis against pH-7.5 buffer, neomycin sulphate (Ethnor Pty, Sydney, 2 mg/ml) and Trasylol (100 units/ml, Bayer, Germany), a kallikrein inhibitor, were added. The initial volume was restored by addition of pH-7.5 buffer, and samples were incubated with substrate at 37 °C for varying periods ranging from 1 to 24 h. The reaction was stopped by refrigeration to —20 °C and the concentration of angiotensin determined at each incubation time using the blood pressure response of the ganglion blocked rat (Peart[12]) and [βAsp(NH_2)^1, Val^5]-angiotensin II, as standard.

Radioimmunoassay. Following dialysis of samples against pH-7.5 buffer, dimercaprol (4 mM) and EDTA (35 mM) were added to inhibit angiotensinases (Haber *et al.*[13]). Initial volume was restored, and samples were incubated with substrate for varying periods of time at 37 °C. The reaction was stopped by addition of an equal volume of ice-cold acetone to the incubate, and the mixture centrifuged for

20 min at 6000 rev./min at 10 °C. The supernatant was diluted 1:10 with bovine serum albumin buffer. To 200 μl of standard solutions of [Asp[1], Ile[5]]-angiotensin I, and to samples 10 μl of antibody and 10 μl of ^{125}I-labelled angiotensin I (5000 counts per 100 s) were added. The mixture was incubated at 5 °C for 16–24 h. Antibody bound ^{125}I-labelled angiotensin was separated from free ^{125}I-labelled angiotensin using dextran-coated charcoal (Herbert *et al.*[14]). From the concentration of angiotensin (ng/ml) at each incubation time an initial-velocity plot was constructed (Lever *et al.*[15]) and the concentration of renin was expressed as the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) after correction for dilution.

Properties of the factor responsible for activation of renin

Amniotic fluid, dialysed to pH 1.5 to destroy renin (Skinner[5]; Lumbers and Skinner[16]) was used to study the properties of the activator of renin at pH 4.5 and pH 7.5, which are above the optimum pH for acid activation of renin.

Two 5.5-ml samples of pooled amniotic fluid were dialysed against pH-1.5 buffer for 24 h at 5 °C; one was subsequently dialysed to pH 4.5, the other to pH 7.5.

Four 5.5 ml-samples of the same amniotic fluid were dialysed against pH-4.5 buffer for 1 h at 30 °C; two samples were mixed; one diluted with an equal volume of pH-4.5 buffer, and to the remaining aliquot an equal volume of amniotic fluid, pretreated to pH 1.5, was added.

Four 5.5-ml samples of the amniotic fluid were dialysed against pH-7.5 buffer for 1 h at 30 °C; two samples were mixed; one diluted with an equal volume of pH-7.5 buffer, and to the remaining fraction an equal volume of amniotic fluid, pretreated to pH 1.5, was added.

Three 5.5-ml samples of the amniotic fluid were dialysed against pH-3.3 buffer for 1 h at 30 °C, two samples were mixed, and one sample diluted with an equal volume of pH-3.3 buffer.

All mixtures were incubated at 30 °C for varying periods at their respective pH, then dialysed to pH 7.5, and the initial velocity of the reaction of renin with sheep substrate determined by bioassay (Fig. 3).

The effect of proteolytic enzymes on the activity of renin

Exogenous pepsin (25 μg/ml) was incubated with three 4 ml-samples of amniotic fluid from different patients at pH 4.5 for 30 min at 37 °C. Samples were subsequently dialysed to pH 7.5 prior to incubation with sheep substrate. The effect of pepsin on amniotic fluid renin was also studied at the pH range 4.5–7.5. The amount of angiotensin generated at pH 7.5 was determined in separate experiments by bioassay and radioimmunoassay (Fig. 4).

Samples of human amniotic fluid were dialysed against pH-7.5 buffer, containing no EDTA. To separate aliquots, the enzymes chymotrypsin, trypsin and carboxypeptidase (EC 3.4.2.1) were added (25 μg/ml, final concentration), and the mixtures incubated at 37 °C for varying periods of time. Further activity of these enzymes was prevented by addition of dimercaprol (4 mM) followed by EDTA (35 mM) and 100 mM DFP. Samples were then incubated with sheep substrate and the concentration of angiotensin formed on incubation determined by radioimmunoassay.

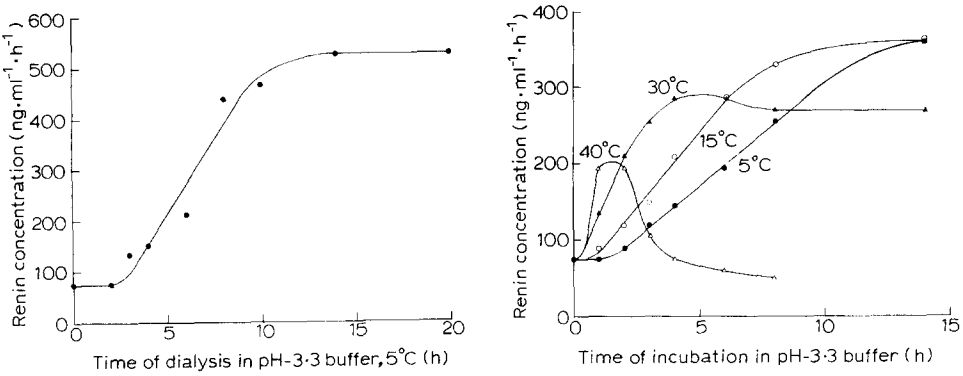

Fig. 1. Effect of time of dialysis in pH-3.3 buffer at 5 °C on the activation of renin in amniotic fluid. Renin concentration is defined as the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) from sheep substrate at pH 7.5 and 37 °C. The concentration of angiotensin formed on incubation was measured by bioassay.

Fig. 2. Effect of temperature on the rate of activation of renin in amniotic fluid dialysed against pH-3.3 buffer at 5 (●) ,15 (○) 30 (▲) and 40 (△) °C. Renin concentration is defined as the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) from sheep substrate at pH 7.5 and 37 °C. The concentration of angiotensin formed on incubation was measured using bioassay.

RESULTS

The rate of activation of renin at pH 3.3 is dependant upon both time and temperature (Figs 1 and 2). The initial slopes of the temperature curves of 5, 15, 30 and 40 °C were 25, 40, 90 and 200 $ng \cdot ml^{-1} \cdot h^{-2}$, respectively (Fig. 2). At 40 °C a decline in the activity of the enzyme was observed with prolonged incubation (Fig. 2). This occurred to a lesser extent at 30 °C. The rate of formation of angiotensin, at pH 7.5, was measured by bioassay.

Properties of the factor responsible for activation of renin

Incubation of pH-1.5-treated amniotic fluid with amniotic fluid at pH 4.5 for 10 h resulted in a progressive increase in the concentration of renin tested by bioassay at pH 7.5 (Fig. 3). In samples to which pH-1.5-pretreated amniotic fluid was not added, a small increase in the activity of renin occurred, but this was much less than in samples incubated with pH-1.5-pretreated amniotic fluid (Fig. 3). The activity of renin in pH-7.5 samples to which amniotic fluid treated to pH 1.5 had been added remained unchanged during incubation (Fig. 3). Furthermore since the activity of renin in these samples was the same as the activity of pH-7.5-treated samples diluted with buffer, no endogeneous renin activity is present. The activity of renin in samples initially dialysed against pH-3.3 buffer was greater than all other samples (Fig. 3).

Effect of proteolytic enzymes on the concentration of renin

Pepsin. Incubation of pepsin with amniotic fluid treated to pH 4.5, resulted in a rise in the activity of renin as tested at pH 7.5, from 300, 550 and 60 $ng \cdot ml^{-1} \cdot h^{-1}$ to 750, 1600 and 200 $ng \cdot ml^{-1} \cdot h^{-1}$, respectively. Activation of renin by pepsin occurred at the pH range 4.5–5.5. No activation was observed at pH 6.5 or 7.5 (Fig. 4). Bioassay was used to measure the rate of formation of angiotensin.

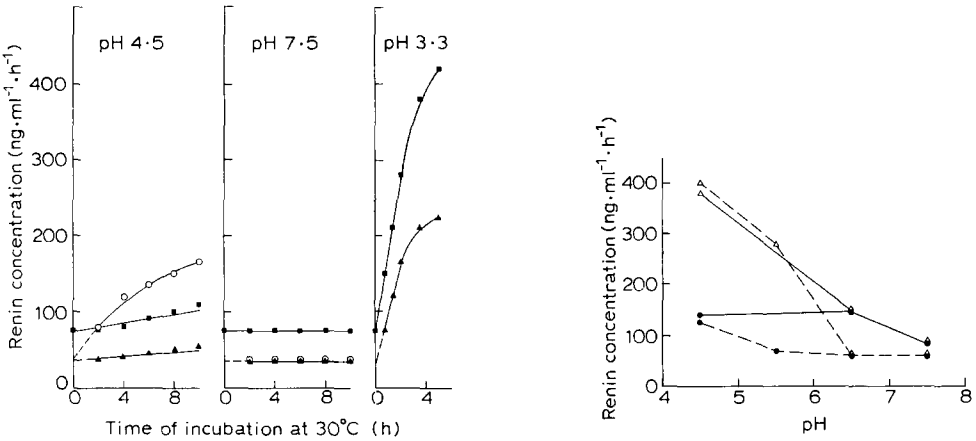

Fig. 3. Effect of incubation of amniotic fluid dialysed to pH 4.5, pH 7.5 and pH 3.3 with an equal volume of amniotic fluid initially treated to pH 1.5 (○), with an equal volume of buffer of pH corresponding to the pH of the sample (▲), or with an equal volume of amniotic fluid treated to the same pH as the sample (■), on the concentration of renin. Renin concentration is defined as the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) from sheep substrate at pH 7.5 and 37 °C. The concentration of angiotensin formed on incubation was measured by bioassay.

Fig. 4. The pH range of activation of renin by pepsin (△) as compared to control (●). Samples were incubated for 1 h at 37 °C. Renin concentration is defined as the rate of formation of angiotensin ($ng \cdot ml^{-1} \cdot h^{-1}$) from sheep substrate at pH 7.5 and 37 °C. ———, bioassay; — — —, radioimmunoassay.

Trypsin. Incubation of amniotic fluid with trypsin at pH 7.5 for 1 h increased levels of renin to 2.1–3.5 times control levels (Fig. 5) as measured by radioimmunoassay.

Chymotrypsin. The increase in activity of renin at pH 7.5 ranged from 1–2 times control levels (Fig. 5), as measured by radioimmunoassay.

Carboxypeptidase. The increase in the activity of renin was 1–1.3 times control levels (Fig. 5). In one sample the activity of renin did not change after 3 h of incubation at pH 7.5, as measured by radioimmunoassay.

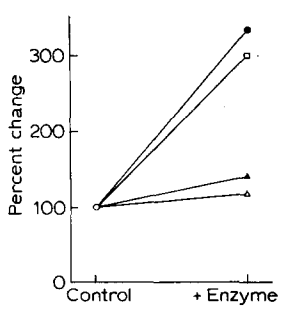

Fig. 5. Mean percentage change in concentration of renin in 3 different samples after 1 h incubation at 37 °C and pH 7.5 with 25 μg/ml of the proteolytic enzymes trypsin (●), chymotrypsin (▲) and carboxypeptidase (△), and in 2 other samples of amniotic fluid incubated with pepsin (□) at pH 4.5. Control expressed as 100% (○). Angiotensin formed on incubation was measured by radioimmunoassay.

DISCUSSION

Prolonged exposure of human amniotic fluid to the pH range 3.3–3.6 increased the activity of renin compared to the activity in samples treated to pH 4.5–7.5 (Lumbers[6]). Since this is not due to more effective inhibition of angiotensinase (Lumbers[6]) and the activity of amniotic fluid renin treated to pH 3.3 is abolished by antibody to human kidney renin (Skinner *et al.*[11]), the increased formation of angiotensin at pH 7.5 is due to increased activity of renin at this pH. The activation of renin at pH 3.3 is dependant upon time and temperature, and the loss of renin activity with prolonged incubations at 40 °C may be due to denaturation of renin at this low pH, and high temperature.

Endogenous renin in amniotic fluid was denatured by pH-1.5 treatment, but the factor responsible for activation of renin was not denatured (Fig. 3). Therefore after pH-1.5 treatment this agent can activate renin at pH 4.5 at a greater rate than in control samples, although to a lesser extent than occurs at pH 3.3. This factor has properties similar to pepsin, an acid-stable enzyme which is denatured at pH values greater than 6.0 (Herriot[17]). Furthermore, exogenous pepsin can activate renin at pH 4.5–5.5 (Fig. 4). The increased rate of formation of angiotensin seen in samples incubated with pepsin at pH 4.5 and pH 5.5 cannot be due to pepsitensin formation (Croxatto and Croxatto[18]; Franze de Fernandez *et al.*[19]), firstly because the activity of renin was tested at pH 7.5, at which pH pepsin is inactive; and secondly because the endogenous substrate concentration in human amniotic fluid is very low (Lumbers[6]). Furthermore, any pepsitensin formed at pH 4.5 would be removed on subsequent dialysis to pH 7.5. Therefore pepsin must act on renin itself, increasing the activity of renin.

Other proteolytic enzymes, trypsin and to a lesser extent chymotrypsin can activate renin on incubation at pH 7.5. The addition of the enzyme inhibitors DFP, EDTA and dimercaprol prevented the subsequent action of these enzymes on either the added substrate or angiotensin formed on incubation. The exopeptidase carboxypeptidase had no effect on the activity of renin.

These findings suggest that activation of renin involves hydrolysis within either the renin molecule or a renin–protein complex. Haas *et al.*[20] and Skeggs *et al.*[7] both suggest that renin is bound to protein. Skeggs *et al.*[7] showed that hog renal renin prepared at pH 7.0, was converted by exposure to pH 2.4 or by prolonged exposure at pH 5.0, to a second form of renin, of higher specific activity but with a similar Michaelis constant. Lumbers[6] also showed that the affinity of renin for substrate was not grossly altered by low-pH treatment. It is suggested that activation of renin therefore involves conversion of an inactive form of the enzyme to an active form.

Preliminary evidence suggests that an inactive form of renin exists in normal male plasma (Lumbers[6]). Two forms of renin may be secreted from the kidney, active and inactive, and stimuli which cause release of active renin may act on intracellular enzymes which hydrolyse inactive renin to an active form. Barajas[21] has suggested that the amorphous character of the mature renin granule is the result of the action of lytic enzymes associated with the Golgi apparatus. Of the cathepsins which are proteolytic, cathepsin D (EC 3.4.4.23) has similar properties to pepsin (Barrett[22]) and cathepsin B1 has been implicated in the activation of trypsinogen to trypsin (Greenbaum *et al.*[23], Otto[24]). Therefore, an intracellular enzyme exists which is present in the

kidney, has a mol. wt of 52 000, is activated by EDTA and which could activate renin. It has been observed that the percentage increase in levels of renin treated to pH 4.5 (plasma renin activity) prior to incubation at pH 7.5 (Skinner[5]) is greater than the increase in levels of renin treated to pH 3.3 (plasma renin concentration) in the same samples, following administration of natriuretic agents (Lumbers[25]). In these experiments, endogenous substrate levels were unchanged. This discrepancy could be due to release of an active form of renin in response to natriuretic therapy. Secretion of renin activated by intracellular enzymes would result in a relatively greater increment in plasma renin activity as compared to plasma renin concentration which in this system measures both active and inactive renin.

Therefore, the activity of amniotic fluid renin is increased by preincubation with the proteolytic enzymes pepsin, trypsin and to a lesser extent chymotrypsin. Furthermore, in human amniotic fluid a factor is present which can increase the activity of renin as tested at pH 7.5 by prior incubation at the pH range 3.3–3.6. This factor is of high molecular weight, since it is not removed by dialysis; is resistant to pH-1.5 pretreatment, and inactive at pH 7.5. Further studies are required to elucidate the nature of this factor and the mechanism by which it modifies the renin molecule, so increasing its activity.

ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council of Australia. E.R.L. is a Senior Research Officer of the N.M. and M.R.C. (Australia).

REFERENCES

1 Turrian, H. (1960) *Helv. Physiol. Pharm. Acta* 18, 259
2 Gould, A. B., Skeggs, L. T. and Kahn, J. R. (1964) *J. Exp. Med.* 119, 389
3 Ferris, T. F. and Mulrow, P. J. (1965) *Clin. Res.* 13, 206
4 Brown, J. J., Davies, D. L. Doak, P. B., Lever, A. F., Robertson, J. I. S. and Tree, M. (1964) *Lancet* 2, 64
5 Skinner, S. L. (1967) *Circ. Res.* 20, 391
6 Lumbers, E. R. (1971) *Enzymologia* 40, 329
7 Skeggs, L. T., Lentz, K. E., Kahn, J. R. and Hochstrasser, H. (1967) *Circ. Res.* 21 (suppl. 11) 91
8 Morris, B. J. and Lumbers, E. R. (1971) *Proc. Aust. Physiol. Pharm. Soc.* 2 (1), 65
9 Hunter, W. M. and Greenwood, F. C. (1962) *Nature* 194, 495
10 Haber, E., Page, L. B .and Jacoby, G. A. (1965) *Biochemistry* 4, 693
11 Skinner, S. L., Lumbers, E. R. and Symonds, E. M. (1968) *Am. J. Obstet. Gynecol.* 101, 529
12 Peart, W. S. (1955) *Biochem. J.* 59, 300
13 Haber, E., Koerner, T., Page, L. B., Kliman, B. and Purnode, A. (1969) *J. Clin. Endocrinol. Metab.* 29, 1349
14 Herbert, V., Lau, K. S., Gottlieb, C. W. and Bleicher, S. J. (1965) *J. Clin. Endocrinol. Metab.* 25, 1375
15 Lever, A. F., Robertson, J. I. S. and Tree, M. (1964) *Biochem. J.* 91, 346
16 Lumbers, E. R. and Skinner, S. L. (1969) *Aust. J. Exp. Biol. Med. Sci.* 47, 251
17 Herriott, R. M. (1962) *J. Gen. Physiol.* 45, 57
18 Croxatto, H. and Croxatto, R. (1942) *Science* 95, 101
19 Franze de Fernandez, M. T., Paladini, A. C. and Delius, A. E. (1965) *Biochem. J.* 97, 540
20 Haas, E., Lamfrom, H. and Goldblatt, H. (1953) *Arch. Biochem. Biophys.* 44, 63
21 Barajas, L. (1966) *J. Ultrastruct. Res.* 15, 400
22 Barrett, A. J. (1971) in *Tissue Proteinases* (Barrett, A. J. and Dingle, J. T., eds), pp. 109–127, North Holland, Amsterdam
23 Greenbaum, L. M., Hirschkowitz, H. and Shoichet, I. (1959) *J. Biol. Chem.* 234, 2885
24 Otto, K. (1971) in *Tissue Proteinases* (Barrett, A. J. and Dingle, J. T., eds), p. 1, North Holland, Amsterdam
25 Lumbers, E. R. (1969) *The quantitation and significance of renin in biological fluids*, M.D. Thesis.