Analysis of Active Renin Heterogeneity (43271)

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Abstract. **Active renin is a heterogeneous enzyme that can be separated into multiple forms with high-resolution isoelectric focusing. The isoelectric heterogeneity may result from differences in glycosylation between the different forms. In order to determine the relationship between active renin heterogeneity and differences in composition or attachment of oligosaccharides, two separate experiments were performed: (i) Tunicamycin, which interferes with normal glycosylation processing, increased the proportion of relatively basic renin forms secreted into the incubation media by rat renal cortical slices. (ii) Endoglycosidase F, which enzymatically removes carbohydrate from some classes of glycoprotein, similarly increased the proportion of relatively basic forms when incubated with active human recombinant renin. In addition, further studies with inhibitors of human renin activity revealed that the heterogeneous renin forms were similarly inhibited by two separate renin inhibitors. These results are consistent with the hypothesis that renin isoelectric heterogeneity is due in part to differences in carbohydrate moiety attachment and that the heterogeneity of renin does not influence access of direct renin inhibitors to the active site of renin.** [P.S.E.B.M. 1991, Vol 197]

Glycosylation of protein is a common posttranslational modification that can create heterogeneity of the resultant glycoprotein (1). Glycosylation may alter the function of some glycoproteins (2), as well as stabilize the tertiary structure of the peptide moiety (3). Furthermore, carbohydrate residues attached to protein can greatly influence the hepatic clearance of the resulting glycoprotein (4).

Renin is an aspartyl protease glycoprotein (5, 6) that is responsible for the formation of angiotensin I from its substrate angiotensinogen. The existence of active renin heterogeneity has been demonstrated in a variety of animals (7–10) and humans (8, 11–14). Since variation of carbohydrate structure and attachment to protein often causes microheterogeneity in glycoproteins (1), it has long seemed likely that active renin heterogeneity may be attributable to variable glycosylation of the enzyme (15).

The present study was designed to test the hypothesis that the isoelectric heterogeneity of renin is due to differences in its carbohydrate moiety and whether the heterogeneity influences binding of renin inhibitors to active renin. We used tunicamycin (TM) to prevent renin glycosylation during synthesis in rat renal cortical slices, and endoglycosidase F (Endo F) to release oligosaccharides from purified recombinant human renin. In each case, the isoelectric heterogeneity of renin was then reexamined. The inhibitory activity of two renin inhibitors (EMD 51 921 and EMD 52 859; E. Merck, Darmstadt, Germany) was also assessed against the major heterogeneous renin forms of intact human plasma renin to determine whether renin heterogeneity influenced the binding of the inhibitors to the active site of the multiple renin forms.

Materials and Methods

Renal Cortical Slices. Eight male Sprague-Dawley rats weighing 250–300 g were used in the TM experiments. One kidney was obtained from each animal following ether anesthesia. Cortical slices approximately 0.4-mm thick were made parallel to the capsule surface. The initial slice was discarded and the next two slices from one hemisphere were paired, so that two pairs were obtained from each kidney.

One pair of slices was washed in 20 ml of physiological salt solution (PSS) (16), containing tunicamycin (10 μg/ml; Sigma Chemical Co., St. Louis, MO), and

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the other served as a control after being washed with PSS only. They were maintained in an oscillating incubation bath (37°C) and bubbled with 95% oxygen and 5% carbon dioxide.

After two 15-min washings, each pair of slices was transferred into a 10 ml flask containing 1.5 ml of incubation media for 1 hr. The composition of the incubation medium was the same as the washing solution. During incubation, the gas phase was layered over the medium to prevent renin inactivation (16).

At the end of the 1-hr incubation, the medium was discarded and replaced with 2.5 ml of fresh medium. The slices were then incubated for an additional hour, after which the medium was collected and immediately frozen at −20°C. The slices from each experiment were homogenized in an ice-cold aqueous solution of bovine serum albumin (BSA, 0.1%), and then centrifuged (2,500*g*, 30 min). The supernatant was kept frozen and later applied to shallow gradient isoelectric focusing gels or assayed directly for renin activity.

Endoglycosidase F and *N*-Glycanase Treatment. Recombinant human prorenin secreted from chinese hamster ovary (CHO) cells transfected previously with a gene encoding human preprorenin was purified (17), trypsin activated (18), and then used as a substrate for endoglycosidase F and *N*-glycanase deglycosylation procedures. Recombinant renin was used as a highly purified source of renin relatively free of contaminating glycoproteins.

Endo F incubations were carried out in initial volumes of 160 μl composed of 100 m*M* sodium acetate buffer (pH = 6.0), 0.1% BSA, 1.25% Nonidet P-40, 15 milliunits of Endo F (Genzyme, Boston, MA), and 0.6 ng, or 2,000 ng of recombinant renin.

N-Glycanase incubations were carried out in initial volumes of 160 μl composed of 200 m*M* sodium phosphate buffer (pH = 7.6), 0.5% BSA, 1.25% Nonidet P-40, 10 units of *N*-glycanase (Genzyme), and 20 ng of recombinant renin.

Both deglycosylation incubations were carried out at 37°C for 0, 1, 3, 5, and 7 days. Control incubations were simultaneously performed in an identical manner without Endo F and *N*-glycanase.

Renin Activity Inhibition of Human Multiple Renin Forms. Plasma from 10 essential, hypertensive, consenting volunteers was pooled and the five major multiple renin forms were separated by shallow gradient isoelectric focusing. The gel segment elution volumes (see below) were 1 ml, and only 100 μl were assayed for renin activity. The remaining 900 μl of each gel segment elution volume from 20 gels were combined so that each of the five major active renin forms were separately pooled. Each of the five pooled elution volumes containing a single multiple renin form served as a source to assess the activity of two renin inhibitors (EMD 52 859 and EMD 51 921). Hypertensive human multiple renin forms were used so as to provide a realistic substrate for the renin inhibitors.

Renin inhibitory activity was measured at renin inhibitor concentrations of 0, 0.25, 0.8, 2.5, 8.0, and 25 n*M* (pH = 6, 37°C). Inhibitory activity for each of the five major multiple renin forms was expressed as the ratio of renin activity (angiotensin I (AI) per milliliter per hour) with inhibitor present, divided by renin activity without the inhibitor, multiplied by 100 (percentage inhibition relative to control). Renin activity without inhibitor corresponded to approximately 5 ng AI/ml/20 min. Renin activity with 95% inhibition was therefore 0.25 ng AI/ml/20 min. Radioimmunoassay concentration ranges for AI were 0.1–10 ng AI/ml.

Isoelectric Focusing and Assay of Renin. Shallow gradient isoelectric focusing of renin was used to quantitate active renin heterogeneity. Isoelectric focusing of rat renin before and after renal cortical slice exposure to TM was carried out as described previously (15) with only minor modifications. Each focusing gel was sliced into 40 segments and eluted in 0.1% BSA, and aliquots were analyzed for renin activity using 40-hr bilaterally nephrectomized rat plasma as a substrate (angiotensinogen) source. The resulting angiotensin I generated was then measured by radioimmunoassay, as described previously (15, 16).

Shallow gradient isoelectric focusing of human recombinant renin before and after exposure to deglycosylation enzymes was done in a similar manner with some modifications. The modified polyacrylamide gels contained 1.92% ampholine (pH 5–7) and 0.08% ampholine (pH 4–6, LKB). The catholyte and anolyte were 0.02 *M* L-histidine and 0.02 *M* L-asparagine, respectively. Focusing was carried out at 20°C; the gels were sliced into 60 segments, and then eluted in 125 μl of 0.1 *M* maleate buffer (pH = 6) with 1% BSA. The entire elution volume was analyzed for renin activity using 24-hr bilaterally nephrectomized sheep plasma as an angiotensinogen source.

Focusing of essential hypertensive human plasma was performed as described for human recombinant renin.

The renin activity corresponding to each isoelectric peak (specific renin form) in a gel was expressed as a percentage of total renin activity recovered from the corresponding gel for the TM data. The limits of detection for AI in the radioimmunoassay are 0.1–10 ng AI/ml. Renin activity peaks in all focusing gels tended to be near, but never above, the upper limit.

Statistical Analysis. Data are presented as mean ± 1 SE. Paired *t* tests were used for the TM data to determine significant differences ($P < 0.05$) between control and TM-treated renal cortical slice renin content, secretory rate, and multiple renin form proportions. For ease of statistical analysis and presentation of the TM data, the values obtained for the proportion

of each form of rat renin were grouped by 2's, i.e., Forms (1 + 2), (3 + 4), and (5 + 6). Differences in enzymatic inhibition of renin activity by renin inhibitors was assessed by estimating the concentration of inhibitor required for 50% and 90% inhibition, and comparing inhibitor concentrations at each inhibition level by analysis of variance.

Results

Effects of TM. The effects of TM on rat renal cortical slice renin release and the proportion of different forms are shown in Tables I and II, respectively. Six forms of rat renin focused consistently at isoelectric pH values of 5.9, 5.7, 5.4, 5.2, 5.0, and 4.8, and were named Forms 1–6, respectively. Neither the rate of secretion of renin into the incubation media nor the content of renin stored in the cortical slice was affected by TM treatment (Table I). TM treatment, however, increased the proportion of the relatively basic Forms [1 + 2] secreted into the incubation media, predominantly at the expense of Forms [3 + 4] (Table II). In contrast to secreted renin, the isoelectric profile of renin remaining in the slices was not affected by TM.

Effects of Endo F and *N*-Glycanase. The effects of Endo F on the isoelectric heterogeneity of human recombinant renin is shown in Figure 1. Five major forms of human recombinant renin were consistently obtained with approximate isoelectric points of 5.7, 5.6, 5.5, 5.2, and 5.1. The actual isoelectric gel renin activity profiles are shown rather than having the peaks grouped by 2's, because Endo F treatment resulted in the appearance of new, relatively basic, renin forms focusing at pH of 6.1 and 5.9. Figure 1 shows the renin isoelectric profiles after 1 and 7 days of incubation with Endo F or vehicle. A substantial leftward shift (relatively more basic isoelectric point) can be seen after Endo F treatment for 24 hr (Fig. 1, Panel A versus B). After 1 week, an additional smaller increase of the new, relatively basic, renin forms can be seen, indicating that the reaction was not yet complete after 24 hr (Fig. 1, Panel C versus D). During the deglycosylation incubation, total renin activity did not differ between Endo F treatment and vehicle control. However total renin activity did decline 20% after 24 hr and 69% after 7 days in both Endo F and control incubations.

Data shown in Figure 1 was collected with 2000 ng of recombinant renin in the Endo F incubation volume. Deglycosylation incubation with 20 ng of renin yielded qualitatively similar results. Additional incubation with only 0.6 ng of renin showed little or no change in the renin isoelectric profile, presumably because too little renin was present as substrate for Endo F.

N-Glycanase had no effect on the isoelectric focusing profile of human recombinant renin. To rule out conversion of renin to a form outside the shallow gradient range, steep gradient isoelectric focusing was also performed (pH 3–10) as described previously for active renin (19). No difference in focusing of renin after *N*-glycanase treatment could be seen with either steep or shallow focusing.

Recombinant Renin and Human Plasma Renin Heterogeneity. Since human recombinant renin heterogeneity may be different compared to normal human plasma renin, the two renin sources were compared after isoelectric focusing. The two renin sources display very similar heterogeneity. Five major renin peaks from both normal human pooled plasma renin and human recombinant renin were recovered with the same isoelectric points.

Effects of Renin Enzymatic Inhibition. The ability of two different renin inhibitors to inhibit human renin enzymatic activity is shown in Tables III and IV. No significant differences were noted for inhibition of the five major isoelectric forms of hypertensive human renin with EMD 51 921 (Table III) or EMD 52 859 (Table IV). EMD 52 859 displayed an IC-50% of ap-

Table I. Renin Release Rate into the Incubation Media and Renin Concentration in the Homogenates of Control and TM-Treated Cortical Kidney Slices

	Control	TM-treated
Incubation media (ngAI/mg/hr/hr)	51 ± 8[a]	45 ± 5
Homogenates (ngAI/mg/hr)	375 ± 50	320 ± 32

[a] Mean ± SE. $n = 8$.

Table II. Effects of TM (10 μg/ml) on the Proportion of Renin Forms in the Incubation Media and in Homogenates of Cortical Kidney Slices

	Forms [1 + 2]	Forms [3 + 4]	Forms [5 + 6]
Incubation media (%)			
Control	70.4 ± 2.3[a]	26.1 ± 2.2	4.6 ± 2.3
TM	77.6 ± 1.9[b]	20.1 ± 1.8[b]	2.3 ± 0.5
Homogenates (%)			
Control	68.6 ± 1.6	24.3 ± 1.4	5.4 ± 0.4
TM	70.8 ± 1.4	23.2 ± 1.4	3.8 ± 0.9

[a] Mean ± SE. $n = 8$.
[b] $P < 0.05$, paired t test between control and TM-treated groups.

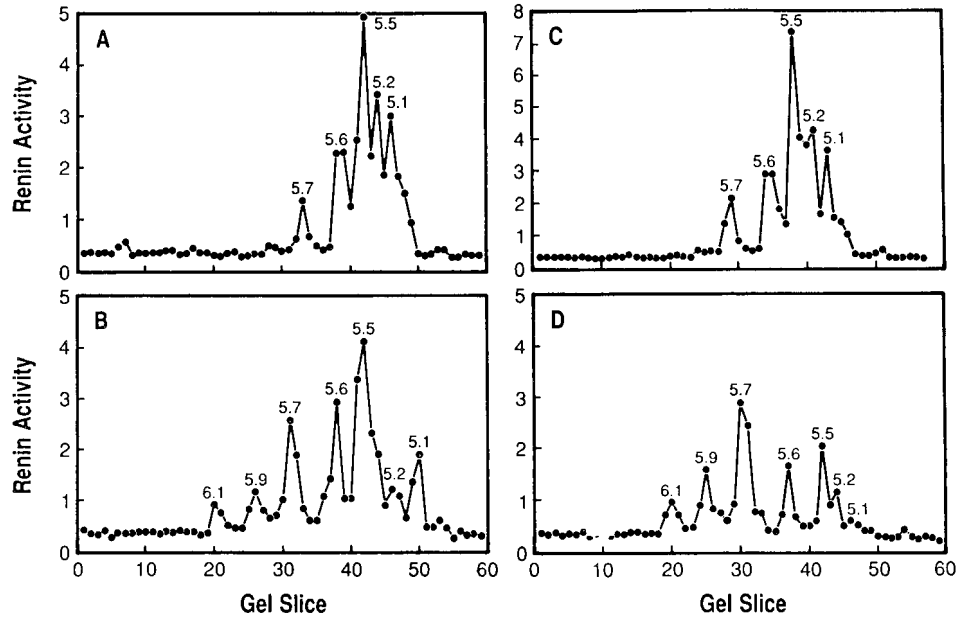

Figure 1. The effects of Endo F on the isoelectric heterogeneity of recombinant renin. After 24 hr of Endo F incubation (Panel B), a substantial leftward shift toward relatively basic isoelectric points can be seen, compared to the 24-hr control (Panel A). After 1 week of Endo F incubation (Panel D), an additional leftward shift is evident. Proportionally, the 24-hr control (Panel A) and 1-week control incubation (Panel C) are very similar, indicating that Endo F is responsible for the shift to relatively basic isoelectric points. Isoelectric points of the five major renin forms are shown above the respective peaks. Renin activity is expressed as ng angiotensin I/ml gel segment eluant/hr assay incubation.

Table III. Percentage of Enzymatic Inhibition of Each of the Five Major Human Renin Forms by Different Concentrations of EMD 51 921

Concentration of EMD 51 921 (n*M*)	Isoelectric points and percentage inhibition of human multiple renin forms				
	5.7	5.6	5.5	5.2	5.1
0.25	4.2 ± 4[a]	13.2 ± 5	7.3 ± 5	14.2 ± 7	14.7 ± 10
0.80	16.1 ± 10	24.3 ± 11	20.0 ± 12	38.1 ± 10	25.2 ± 11
2.50	58.8 ± 18	63.1 ± 11	66.7 ± 11	67.8 ± 12	60.1 ± 17
8.00	88.9 ± 5	89.1 ± 5	93.8 ± 3	94.0 ± 2	81.8 ± 8
25.0	99.8 ± .1	99.5 ± 1	98.2 ± 1	98.4 ± 1	99.1 ± 1

[a] Mean ± SE. $n = 4$.

proximately 5×10^{-9} *M*, while EMD 51 921 was somewhat more potent, having an IC-50% of approximately 2×10^{-9} *M*.

Discussion

The purpose of this study was to determine (i) whether the isoelectric heterogeneity of active renin is related to the carbohydrate moieties attached to renin, and (ii) whether the heterogeneity of active renin is important for renin enzymatic inhibition.

Tunicamycin blocks the addition of *N*-acetylglucosamine-1-phosphate to dolichol phosphate, the first step in the eventual addition of oligosaccharide to certain asparagine residues, leading to glycoprotein formation. In this study, TM shifted the proportion of secreted renin forms, decreasing the relatively acidic forms and significantly increasing the proportion of the relatively basic forms. Although TM caused a significant shift in the secreted renin forms, the shift was not large. This is probably because only some of the basal-secreted active renin is newly synthesized (19, 20) and thus available for modification by TM. In addition, the *de novo* synthesis of renin was small since only 1% of the renal renin content is normally synthesized or secreted per hour (19), and renin is only present in picomolar quantities in plasma. Since a component of basal active renin secretion is newly synthesized (constitutive pathway) and relatively acidic (19), it appears that TM interferes with the newly synthesized acidic components of secreted renin, and elicits a fall in the secretion of the relatively acidic forms (thereby increasing the proportion of the relatively basic secreted renin forms). TM did not significantly alter the isoelectric heterogeneity of renin in the renal homogenate, presumably because the renal renin storage pool is approx-

Table IV. Percentage of Enzymatic Inhibition of Each of the Five Major Human Renin Forms by Different Concentrations of EMD 52 859

Concentration of EMD 52 859 (n*M*)	Isoelectric points and percentage inhibition of human multiple renin forms				
	5.7	5.6	5.5	5.2	5.1
0.25	1.0 ± 3[a]	−3.0 ± 7	7.8 ± 4	11.5 ± 3	4.1 ± 4
0.80	−1.5 ± 4	5.8 ± 5	6.7 ± 3	7.8 ± 2	4.9 ± 2
2.50	16.5 ± 9	21.5 ± 6	23.6 ± 8	24.2 ± 5	26.6 ± 9
8.00	75.9 ± 4	79.2 ± 3	76.2 ± 5	80.2 ± 3	75.1 ± 6
25.0	99.2 ± 1	99.6 ± 3	97.7 ± 2	94.9 ± 2	96.8 ± 2

[a] Mean ± SE. $n = 4$.

imately 10- to 100-fold greater (21, 22) than the small amount of renin synthesized during incubation with TM.

Endoglycosidase F hydrolyzes the glycosidic bonds of the *N,N*-diacetylchitobiose core structure of many high-mannose and bi-antennary asparagine-linked oligosaccharides. Endo F caused a leftward shift in the isoelectric focusing profile of human recombinant renin, leading to the apparent formation of new relatively basic renin forms. Presumably, the deglycosylation of recombinant renin resulted in the removal of acidic carbohydrate moieties, which are partly responsible for the overall isoelectric point of the multiple renin forms. The hydrolysis made little additional progress after the initial 24-hr period, and after 7 days some renin had still not apparently shifted leftward (relatively basic). Normally, sodium dodecyl sulfate-denatured glycoproteins are far easier to deglycosylate than the native glycoprotein. However, sodium dodecyl sulfate-denaturing causes destruction of renin activity, and was therefore not employed. It is, therefore, likely that complete deglycosylation was not achieved. In addition, some of the renin forms may not have been appropriate substrates for Endo F, or Endo F may have lost its activity during the incubation. Carbohydrate moieties of some, but not all, rat renal renin appear to be resistant to Endo F cleavage (23). Interestingly, trypsin-activated renin derived from a human chorionic trophoblast culture showed a similar shift in microheterogeneity when exposed to Endo F (24).

N-Glycanase did not cause any noticeable change in the isoelectric profile of renin, despite its broader specificity. This was probably because SDS conditions were not employed and the enzymatic deglycosylation was attempted at pH 7.6, despite an *N*-glycanase pH optimum of approximately 8.6. This was done to prevent the near total destruction of renin activity seen at pH 8.6 over the time course of the incubation.

Tunicamycin, Endo F, and *N*-glycanase only affect *N*-linked glycoproteins. Therefore, the possibility that active renin heterogeneity may be due to *O*-linked glycosylation was not assessed. However, human renin is commonly thought to be glycosylated at two potential *N*-glycosylation sites (25). Treatment with a sialidase enzyme to remove negatively charged terminal sialic acid was also not employed, since remaining sugars might influence the isoelectric point. Neuraminidase treatment did, however, decrease the microheterogeneity of chorionic activated renin (24).

In order to ensure that human recombinant renin heterogeneity was similar to the heterogeneity displayed by human plasma renin, the two different renins were compared on isoelectric focusing gels. Both renin types focused with the same multiple isoelectric points. The human recombinant renin was a trypsin-activated secretory product of a Chinese hamster ovary cell line transfected with a human preprorenin containing plasmid (17, 18). If the isoelectric heterogeneity of active renin is due to posttranslational modifications such as glycosylation, then the CHO cell performs similar modifications to renin as the human juxtaglomerular cell responsible for human plasma renin. The CHO cell line has been shown to express prorenin in at least two forms, with differing glycosylation (26).

Direct enzymatic inhibition of the human multiple renin forms was used to probe the active site of the different isoelectric forms of human renin. All five major forms of human active renin were equally inhibited, as demonstrated by two renin inhibitors, indicating that the heterogeneity does not influence the active site of the enzyme. This demonstration does not provide direct evidence that renin heterogeneity is due to glycosylation. However the specific activities of renin in the rat (6) and human (11) also appear to be independent of isoelectric heterogeneity. Finally, the *Km* for recombinant renin before and after deglycosylation with Endo F (18) is also essentially identical. Therefore, the isoelectric heterogeneity does not appear to play a role in the direct enzymatic function of renin, nor does the glycosylation of renin.

Glycosylation and isoelectric heterogeneity of renin share other common aspects. Rat renin has been shown to have at least three differently glycosylated forms, each possessing a different hepatic clearance rate (23). Rat renin also displays isoelectric heterogeneity (11), with the relatively basic forms exhibiting greater clear-

ance (hepatic) rates (27). This has also been demonstrated for dog renin (28). Since both isoelectric heterogeneity (27) and degree of glycosylation (23) can determine the hepatic degradation of renin, it is likely that renin isoelectric heterogeneity is due, at least in part, to glycosylation.

The isoelectric heterogeneity of active renin is related to the glycosylation of renin, since TM changed the secreted renin isoelectric profile from rat kidney slices, and Endo F altered the renin isoelectric profile of recombinant renin. In addition, both isoelectric heterogeneity and glycosylation determine the hepatic clearance of renin. Alternatively, some of the isoelectric heterogeneity of renin could be due to differences in processing of renin mRNA or translated protein (29). Finally, renin enzymatic inhibition appears to be independent of isoelectric heterogeneity.

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